The intestinal microbiome of farmed rainbow trout
Oncorhynchus mykiss (Walbaum)

**THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING FOR
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BY**

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Dedication

To my Dad, who introduced me to fly fishing. I'm a long way from the shores of Lough Mask now.

To my uncle, Fr. Kevin Lyons and my grandmother, Mrs. Edith 'Did' Daubney.

Declaration

I hereby declare that the work and results presented in this thesis have been composed in their entirety by me. Except where specifically acknowledged, the work described in this thesis has been conducted by me and has not been submitted for any other degree or qualification.

Signature: _____

Signature of supervisor: _____

Date: _____

Abstract

The study of the gut microbiota of fish began in the 1930's and since that time a considerable amount of information has been collated on its composition and diversity. These studies have revealed that the microbial communities of the fish gastrointestinal tract are generally difficult to culture on bacteriological media and mainly consist of bacteria, archaea, viruses, yeasts and protists. The bacteria appear to be the most abundant of these microbial groups and their activity may have major implications for host health, development, immunity and nutrition. Therefore, much of the most recent published research has focused on developing improved methods of identifying the extent of the bacterial diversity within the fish gut and unravelling the potential influence of these microorganisms on the health of farmed fish species. However, whilst such studies have improved our knowledge of the dominant bacterial groups present in the rainbow trout gastrointestinal tract, the limited resolution capacity of many of the methods used has meant that our understanding of their baseline composition in healthy fish remains poorly understood.

In this study, the bacterial communities that inhabit the intestine, now commonly referred to as the 'microbiome', of farmed Rainbow trout (*Oncorhynchus mykiss*) were characterized using a culture independent high-throughput molecular sequencing method. The microbiome of the intestinal lumen and mucosa was investigated to ascertain the true extent of the bacterial diversity present in this fish species prior to further experiments. It was found that the diversity of the intestinal microbiome was greater than previous studies had reported with a total of 90 and 159 bacterial genera being identified in both the lumen and mucosal regions respectively. The dominant bacterial phyla identified in both of the regions investigated were Proteobacteria, Firmicutes, Fusobacteria, Bacteroidetes and Actinobacteria. Furthermore, the data collected suggested that the intestinal microbiome may be similar in structure between individual fish, and illustrate the utility of next generation molecular methods in the investigation of the fish gut microbiome.

A study was conducted to examine the effect of diet on the composition of the intestinal microbiome of rainbow trout. Two diets, one control and one treatment, were prepared which were identical apart from that the treatment diet contained a microalgal component at 5% of the total formulation. These diets were fed to rainbow trout for a total of 15 weeks. At the end of the trial period a total of 12 fish, three from each of four tanks, were sacrificed from each of the control and treatment groups and their intestinal tissue was sampled in order to compare the composition of the microbiome of both groups. The results revealed that both groups of

fish shared similar microbiome compositions, with the Tenericutes being by far the most dominant phylum observed. The structure of the intestinal microbiome was not significantly different between both populations of trout tested. An increased level of bacterial diversity was noted in the treatment fish, however, this was not found to be statistically significant. A limited number of bacterial taxa were discriminatory between diets and were significantly elevated in the treatment group. These taxa were predominantly lactic acid bacteria of the genera *Streptococcus*, *Leuconstoc*, *Lactobacillus*, *Lactococcus* and *Weissella*. The results of this study suggested that the minor difference in the diets fed resulted in a correspondingly minor alteration in the intestinal microbiome of the tested rainbow trout. This may indicate that diet composition can modify the composition of the intestinal microbiome of these fish.

A further study was conducted to investigate the structure of the intestinal microbiome from groups of fish reared in both freshwater cages and aquarium systems, in order to assess whether or not fish raised in different environments share similar microbiomes. This study also employed a novel computational tool, PICRUSt, to analyse the predicted functional capacity of the microbial communities of individual fish sampled from both environments. The data collected suggested that the structure of the intestinal microbiome was similar regardless of where the fish were raised, with the Tenericutes, Firmicutes, Proteobacteria, Spirochaetae and Bacteroidetes representing the dominant bacterial phyla recorded in the rainbow trout intestine. This suggests that the host may regulate the formation of the intestinal microbiome. A significant difference was however noted in community membership between the fish populations tested, which may point to an environmental influence on the intestinal microbiome. These data suggest that both deterministic host factors and stochastic environmental influences play important roles in shaping the composition of the bacterial communities in the intestine of these fish. The PICRUSt analysis revealed that gene pathways relating to metabolism, transport and cellular processes were enhanced in all of the fish studied, which may signal an involvement of these communities in the digestive processes of rainbow trout.

In conclusion, this study used high-throughput sequencing methods in order to improve our understanding of the intestinal microbiome of farmed rainbow trout, and the effect of dietary and environmental factors on its composition. This research has generated scientific information relating to baseline bacterial community compositions in healthy fish, which may be used in future experiments including screening these baselines against the effects of novel aquafeed formulations, environmental perturbations or pathogenic challenges.

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Conferences attended and work presented

Aquaculture Europe 2016 'Food for Thought', Edinburgh International Conference Centre, Edinburgh, UK, 20th-23rd September 2016. Oral presentation: Phylogenetic and functional characterization of the distal intestinal microbiome of farmed Rainbow trout *Oncorhynchus mykiss*.

British Fish Veterinary Society Spring Conference, Norton House Hotel, Edinburgh, 22nd-23rd March 2016. Poster: Phylogenetic and functional characterization of the distal intestinal microbiome of rainbow trout *Oncorhynchus mykiss* from both farm and aquarium settings. Poster awarded 2nd place.

University of Stirling Institute of Aquaculture, Lunchtime Seminar, January 28th 2016. Oral Presentation: The intestinal microbiome of farmed rainbow trout *Oncorhynchus mykiss*

Alltech Aquaculture Workshop, Forayar Hotel, Streymoy, Faroe Islands 19th-20th November 2015. Oral presentation: Exploring the rainbow trout *Oncorhynchus mykiss* intestinal microbiome using next generation sequencing.

The UK Probiotics Conference 2015 'Microbes and Microbiomes: The Gut Feeling', Royal Holloway, University of London, 29th June – 1st July 2015. Oral presentation: Exploring the microbial diversity of the distal intestinal lumen and mucosa of farmed rainbow trout, *Oncorhynchus mykiss*, using next generation sequencing. Book of Abstracts, pg. 27.

World Aquaculture Society (WAS) 2015 'Aquaculture for Healthy People, Planet and Profit', Jeju Exhibition and Convention Centre, Jeju, South Korea 26-30th May 2015. Oral presentation: Exploring the diversity of the rainbow trout *Oncorhynchus mykiss* intestinal microbiome using next generation sequencing.

University of Stirling Institute of Aquaculture, 4th PhD Research Conference on Sustainable Aquaculture: 'Looking Forward', 18th February 2015. Poster: Exploring the microbial diversity of the rainbow trout *Oncorhynchus mykiss* distal intestine using next generation sequencing.

Alltech Biotechnology Inc. 30th International Feed Symposium 'What If?', Lexington, Kentucky, USA, 18-21 May 2014. Poster: Intestinal microcommunities in farmed rainbow trout *Oncorhynchuis mykiss*.

Alltech Biotechnology Inc., Research Seminar Series, Dunboyne, Dublin, Rep. of Ireland, 7th May 2014. Oral presentation: The intestinal microflora of farmed rainbow trout *Oncorhynchus mykiss*.

European Association of Fish Pathologists (EAFP) 16th International Conference on Diseases of Fish and Shellfish, Tampere, Finland, 2-6 September 2013. Poster: Intestinal microcommunities in farmed rainbow trout *Oncorhynchus mykiss*. Book of Abstracts pg. 214

Alltech Biotechnology Inc., Research Seminar Series, Nicholasville, Kentucky, USA, 19th February 2013. Oral presentation: Improving the health of farmed fish.

Publications related to this thesis

Lyons, P.P., Turnbull, J.F., Dawson, K.A. and Crumlish, M. (2015) Exploring the microbial diversity of the distal intestinal lumen and mucosa of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) using next generation sequencing (NGS). *Aquaculture Research* **doi:** 10.1111/are.12863

Lyons, P.P., Turnbull, J.F., Dawson, K.A. and Crumlish, M. (2016) Effects of low-level dietary microalgae supplementation on the distal intestinal microbiome of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research* **doi:** 10.1111/are.13080

Lyons, P.P., Turnbull, J.F., Dawson, K.A. and Crumlish, M. (2016) Phylogenetic and functional characterization of the distal intestinal microbiome of rainbow trout *Oncorhynchus mykiss* from both farm and aquarium settings. *Applied Microbiology* (In Press).

Table of Contents

List of figures

List of tables

List of abbreviations

AMOVA   Analysis of Molecular Variance

ANOVA   Analysis of Variance

BCFA   Branched Chain Fatty Acids

BLAST   Basic Local Alignment Search Tool

CFU   Colony Forming Units

DGGE   Denaturing Gradient Gel Electrophoresis

DNA   Deoxyribonucleic Acid

FCR   Feed Conversion Ratio

GI   Gastrointestinal

HTS   High Throughput Sequencing

KEGG   Kyoto Encyclopedia of Genes and Genomes

LAB   Lactic Acid Bacteria

NFE   Nitrogen Free Extract

NSTI   Nearest Sequenced Taxon Index

OTU   Operational Taxonomic Unit

PBS   Phosphate Buffered Saline

PCoA   Principal Coordinate Analysis

PCR   Polymerase Chain Reaction

PICRUSt   Phylogenetic Investigation of Communities by Reconstruction of
                 Unobserved States

PTS   Phosphotransferase System

rDNA   Ribosomal Deoxyribonucleic Acid

RDP   Ribosomal Database Project

RNA   Ribonucleic Acid

rRNA   Ribosomal Ribonucleic Acid

SCFA   Short Chain Fatty Acids

SD   Standard Deviation

SEM   Standard Error of the Mean

SGR   Specific Growth Rate

STAMP   Statistical Analysis of Metagenomic Profiles

TSA   Tryptone Soy Agar

TVC   Total Viable Count

Chapter 1.

General introduction and literature review

1.1. Rainbow trout aquaculture

Rainbow trout are ray finned teleost fish in the family Salmonidae of an elongate, fusiform body shape. Their colour can vary according to habitat, size and maturity. They generally present with a blue to olive green colour above a pink band that extends along the lateral line with a white underbelly. They possess a forked tail, paired pelvic and pectoral fins, single anal and dorsal fins and an adipose fin (Fig 1.1). The species was originally designated *Salmo mykiss* by German naturalist and taxonomist Johann Julius Walbaum in 1792, based on specimens collected from the Kamatchka Peninsula in Russia. Sir John Richardson, a Scottish naturalist, named a specimen of this species *Salmo gairdneri* before it was later corrected to *Salmo irideus*. Both of these names were however deemed inappropriate once it was determined that Walbaum's type description was conspecific. DNA genotyping has since shown that rainbow trout are more closely related to Pacific salmon (*Oncorhynchus* species) than to Brown trout *Salmo trutta* or Atlantic salmon *Salmo salar*. The genus was therefore changed and is now known as *Oncorhynchus mykiss* (Walbaum).
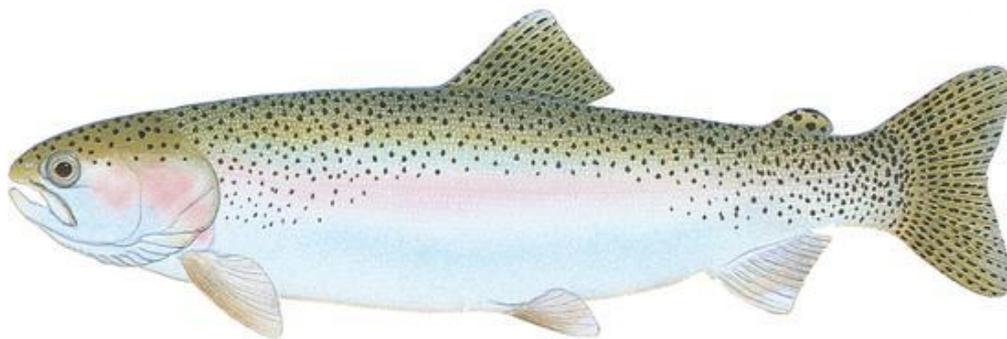
Figure 1.1. Illustration of a rainbow trout (*Oncorhynchus mykiss*) depicting the principal colouration and morphological features of this fish species. Image available in public domain (http://lake-link.com/fish/id.cfm/25/Rainbow-Trout).

Rainbow trout are native to the Pacific drainages of North America, ranging from Alaska to Mexico, the Kamatchka Peninsula in Russia and the Okhotsk Sea (Behnke 1992). Since 1874, this species has been introduced to all waters on all continents except Antarctica, for recreational angling and aquaculture purposes (Cowx 2005). In the wild, the diet of adult rainbow trout consists of insects, molluscs and crustaceans, especially freshwater shrimp.

Rainbow trout are ideally suited to aquaculture for a variety of reasons. They are a robust, easy to spawn and fast growing fish species. Furthermore, they are tolerant of handling, thrive in a wide range of environments and salinities, and their large fry are easily weaned on to artificial pelleted feeds. Fry are traditionally reared in small fibreglass or concrete tanks, before being transferred to larger net pens or ponds when they reach 10 cm in length. Once adapted to formulated diets, the pellet size can be increased in accordance with the size of the fish, up to their final harvest weight, which ranges from 500 g to > 1.2 kg. Formulated feeds for rainbow trout principally contain fish meal, fish oil, grains, vitamins and minerals. The inclusion level of fish meal has been reduced in recent years in favour of more sustainable sources of protein such as plant-based ingredients (e.g. soybean meal). Generally, dietary protein constitutes approximately 35-45% of the total feed composition and dietary fat levels often exceed 20% in high energy feeds (Sedgewick 1990, Cowx 2005). These diets are converted efficiently by rainbow trout, and food conversion ratios (FCR) average 1:1 (Morales et al 1994). Farmers favour using hormonal sex-reversal to produce all female stock, as males tend to mature more rapidly (at approximately one year) which can devalue the final quality of the fillet. Female rainbow trout will usually reach maturity much later, at between two and three years of age, and exhibit better growth rates and resistance to handling stress (Sheehan 1999, Cowx 2005). In addition to a range of food products, farmed rainbow trout are used to stock recreational game fisheries and for the sale of fry and eggs to other commercial farms and research facilities.

Rainbow trout aquaculture began in the late nineteenth century, and increased in significance in the early 1900's when the Jøker family from Denmark designed a farm whereby fresh water flowed through each consecutive pond. This layout radically improved the farm output whilst optimizing water usage. The same family, under the ownership of L Svend Jøker, opened the first trout farm producing 'table fish' of about 500 grams in Lincolnshire, United Kingdom in 1948 (British Trout Association 2016). There are now roughly 360 rainbow trout farms in the UK, with a total production of approximately 16,000 metric tonnes per annum (British Trout Association 2016). The 1950's saw the advent of pelleted fish feeds, which was a game changer for the industry. Since then, rainbow trout aquaculture has grown exponentially, with farms becoming established worldwide and a total production of approximately 900,000 tonnes in 2016 (Figure 1.2). The principal regions of production are presently located in Europe, North America, Japan, Australia and South America. The greatest production of rainbow trout comes from Chile and Norway (Tveteras et al 2015), where fish are now ongrown to large sizes at offshore marine sites. Iran, Turkey, Italy, the USA, Denmark, France and the UK also farm

this species intensively, but the majority of these farms grow small, or 'table' sized fish inland using traditional freshwater flow-through aquaculture systems.

a)

Large Rainbow Trout
Production by country

b)

Small Rainbow Trout Production by Country
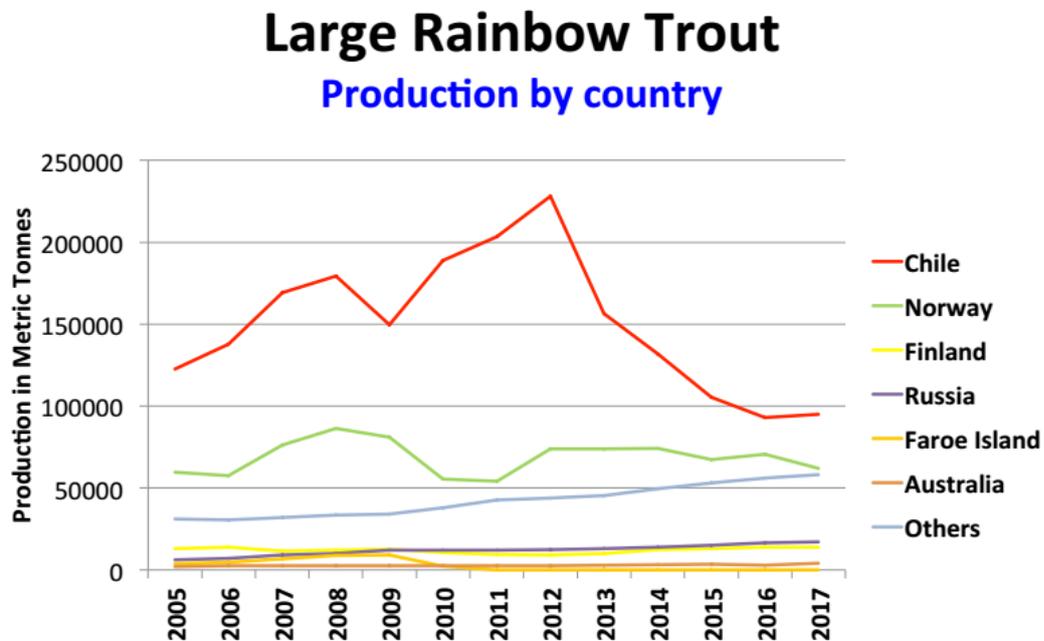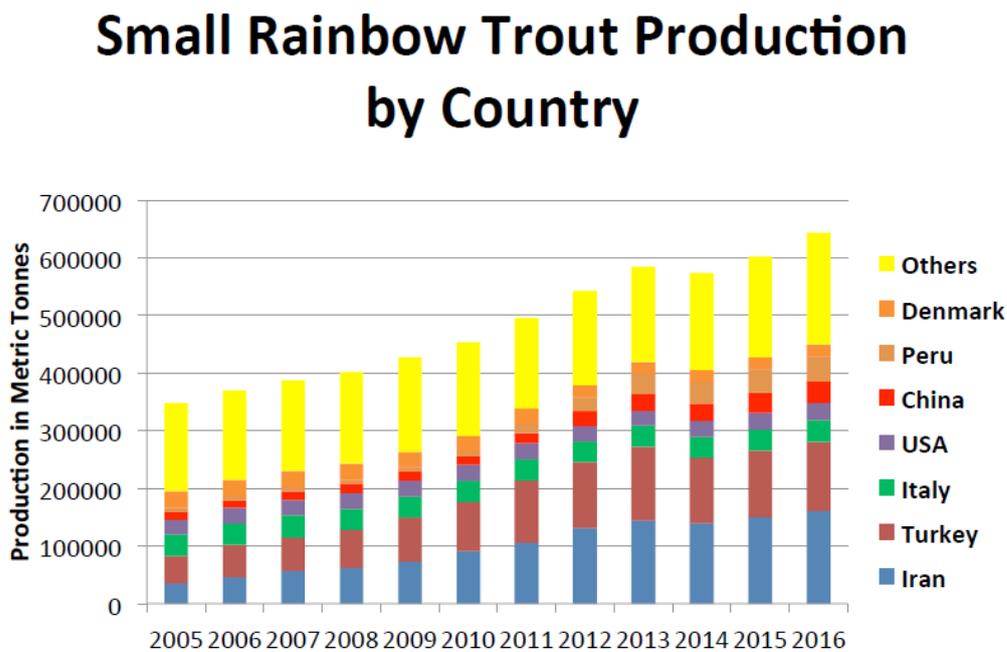
Figure 1.2. Total **a)** large (> 1.2 kg) and **b)** small (<1.2 kg) rainbow trout (*O. mykiss*) aquaculture production by country (Tveteras et al 2015).

3

1.2. The morphology and function of the teleost fish intestine

Teleost fish can be placed into four categories according to their respective feeding habits: detritivores, herbivores, omnivores and carnivores. The gastrointestinal (GI) tract morphology varies across these four groups. In fish that feed on detritus or plant-based ingredients, the intestinal tract tends to be quite long, and these fish spend a lot of time feeding in order to meet their energy and nutrient requirements (Dimitroglou 2009). The greater length of the intestine in herbivorous fish has also been posited to aid in additional processing of relatively difficult to digest food items (Horn 1997, Clements & Raubenheimer 2005). In contrast, omnivorous fish have shorter intestines, and carnivorous fish have the shortest GI tract out of the four categories. Carnivorous farmed fish species are often fed on a high protein fish-meal based diet and their gut morphology reflects this, in that they possess a stomach, pyloric caecae and a short intestine, which is divided into anterior and posterior regions (Figure 1.3). Correspondingly, abilities to digest protein are well developed, but carbohydrate digestion is low compared with omnivorous and herbivorous fish (Buddington et al 1997).
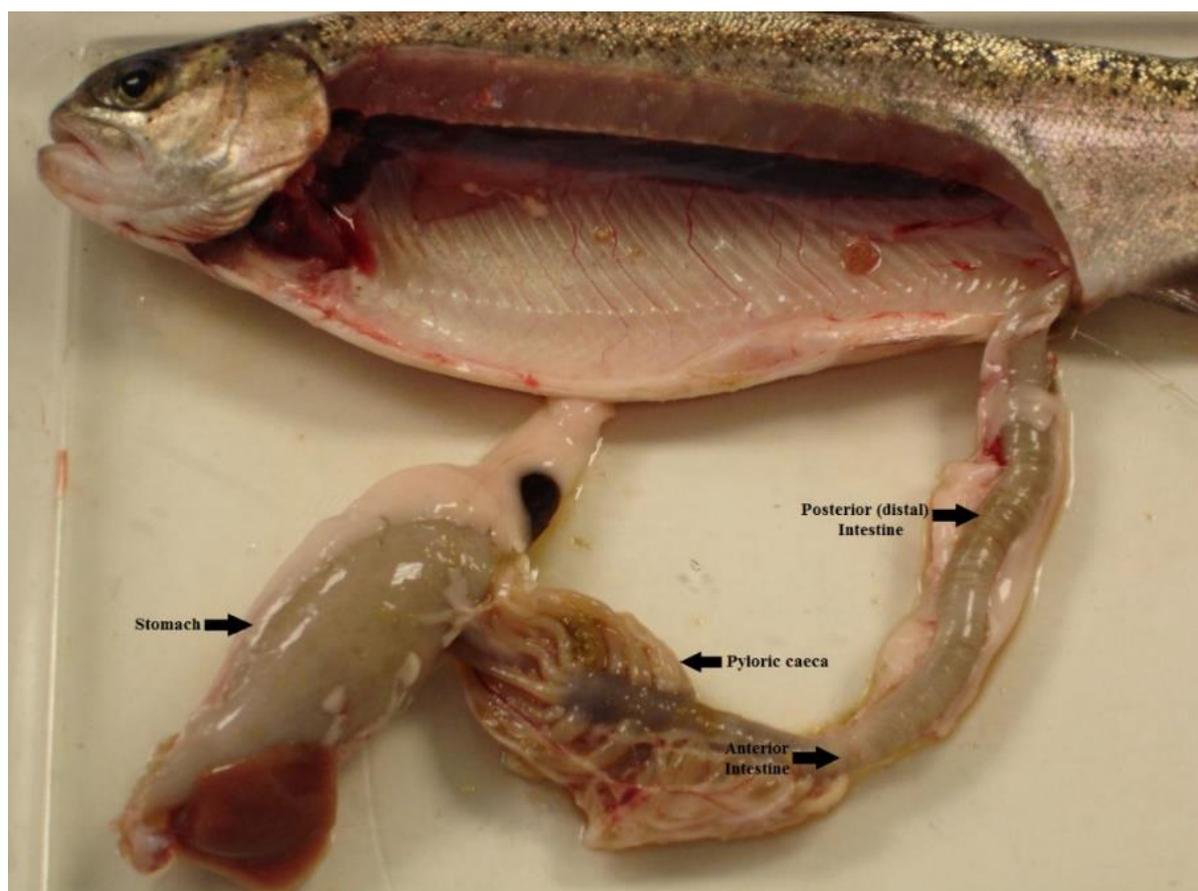
Figure 1.3. The rainbow trout gastrointestinal tract. Stomach, pyloric caeca, anterior and posterior (distal) intestine are shown (arrows). (D. Merrifield, personal communication, 6th February 2013).

As in endothermic animals, the teleost stomach secretes hydrochloric acid which lowers the pH to a level of 2-4, to aid in the digestion of a high protein diet. The pyloric caeca are blind-ending pouches that extend outward from the pylorus close to where the intestine leaves the stomach, and are only present in fish with stomachs. These finger-like projections, which can number from tens to hundreds, serve to increase the absorptive surface area in the GI tract of fish. More recently, it has been suggested that the pyloric caeca may act as fermentation chambers that are involved in lipid metabolism in the European seabass *Dicentrarchus labrax* (Sun et al 2013). In between the pyloric caecae are layers of fat that house the pancreas. The pancreas secretes a variety of digestive enzymes such as trypsin, chymotrypsin, elastase and collagenase. In the intestine, the pH rises along its length to a level of 7-8 and facilitates the digestion of proteins, fats and carbohydrates (Ringø et al 2003). Proteins and carbohydrates are hydrolysed by the pancreatic enzymes, producing peptides and disaccharides which are hydrolysed further by peptidases and disaccharidases embedded in the apical membrane of intestinal enterocytes (Buddington et al 1997). The activity of the pancreatic enzymes declines from the proximal to the distal intestine, suggesting that the contribution of the indigenous intestinal microflora may be of particular importance in the distal region, where feed components that have escaped digestion in the upper GI tract may be absorbed. The resulting sugars and amino acids are then transported into the enterocytes by integral membrane transporters. In herbivorous species, the intestine is solely responsible for the digestion of feed as these fish (e.g. carp, parrotfish) often lack a stomach and pyloric caeca. Herbivorous and detritivorous fish may have the ability to triturate their feed which increases the efficiency of feed digestion and fermentation by the intestinal microbial communities present along the intestine (Dimitroglou 2009, Dimitroglou et al 2011). In omnivorous fish species, either a stomach or a long intestinal tract, or both (e.g. sea bream, sole) are present as an adaptation to their dynamic feeding habits.

The main function of the teleost fish intestine, as with other animals, is to digest and absorb nutrients. However, it is also involved in water and electrolyte balance, endocrine regulation of digestion and metabolism, and immunity (Ringø et al 2003). The intestine of teleost fish consists of a simple, columnar absorbing epithelium of villi, the surfaces of which are covered with cells called enterocytes. The surface of the enterocytes is in turn lined with further folds, or microvilli, which serve to further enhance digestive capacity (Figure 1.4). The microvilli membrane contains enzymes, transporter proteins and ionic channels, all enclosed within a lipid bilayer. Digestive enzymes are bound within the enterocyte cell membrane and are not

secreted. However, these enzymes can become active in the intestinal lumen if they are sloughed from the end of villi. Enterocytes are joined together at the apical end of the lateral surface by junctional complexes, consisting of anchoring adhesion belts and desmosomes. Located closest to the lumen are the occluding tight junctions (Jutfeld 2006). These tight junctions protect against the possible translocation of large molecules and opportunistic bacteria.
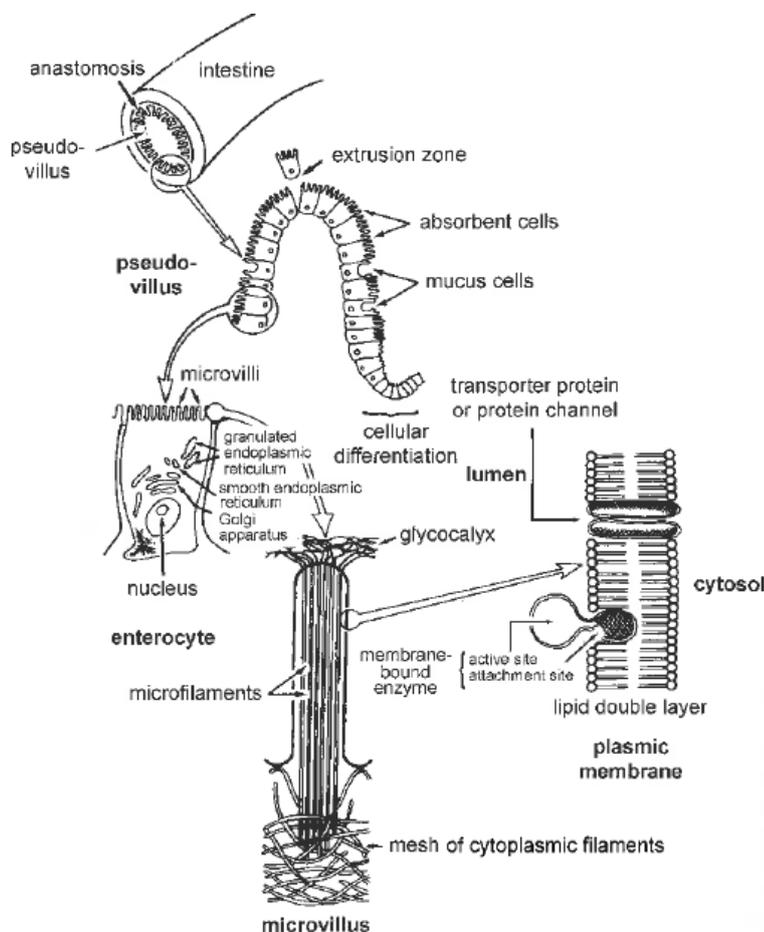
Figure 1.4. The anatomy and histology of the teleost fish intestine, including molecular structure of the microvilli plasma membrane (from Guillaume & Choubert 1999).

In the mid-portion of the intestinal enterocytes, there are numerous relatively large endocytic vacuoles (0.5-1µm in diameter), containing absorbed fat, carbohydrate and/or protein molecules. Enterocytes are produced at the basement membrane of the villi, before migrating to the perimeter, where they are constantly replaced (Buddington & Kuz'Mina 2000). In this way, villi length is dictated by how quickly enterocytes are replaced. Running through the centre of the villi is the lamina propria, which contains arterioles and venules that connect to a portal vein, which transports absorbed molecules from the basement membrane to the liver for nutrient storage, metabolism, and detoxification. Immune cells such as macrophages and

granulocytes also reside in the lamina propria and mucosa and function as antigen processing cells (Buddington et al 1997, Pettersen 2003) for T and B lymphocytes. Whilst fish lack lymph nodes and Peyer's patches, they do possess epithelial cells that share morphological similarities with mammalian M-cells and may carry out similar antigen sampling functions (Fuglem et al 2010). In addition to enterocytes, goblet cells are present in the mucosal folds, and function in the production of mucous that helps to bind opportunistic pathogens, preventing them from colonizing the intestinal epithelium (Figure 1.5).
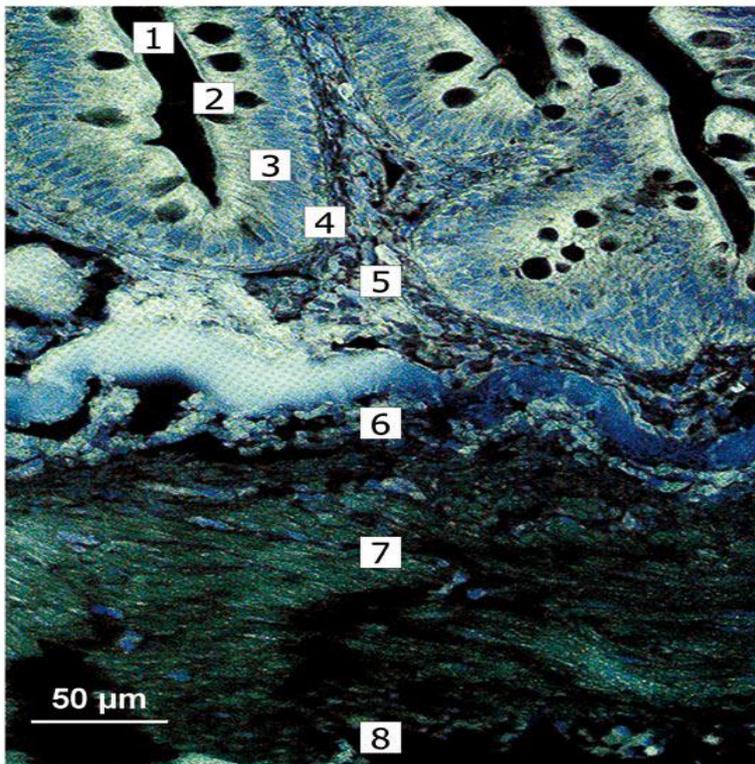
Figure. 1.5. Cross sectional view of the rainbow trout intestine using fluorescence microscopy. Layers of the intestinal wall: 1-Lumen, 2-Goblet cells, 3-Enterocyte, 4-Basal membrane, 5-Lamina propria, 6-Sub mucosa, 7-Circular and longitudinal muscle layer, 8-Serosal layer (from Jutfeld 2006).

1.3. The intestinal microflora of fish

1.3.1. Background

The complex communities of microorganisms that inhabit body sites exposed to the environment have traditionally been referred to as the 'microflora'. In recent years this term has been revised and the communities are now more frequently referred to as the microbiota or microbiome. The GI tract of animals is populated by a diverse and complex microbiome, where the Bacteria are the dominant group, and is hypothesized to play an important role in nutrition, physiology and health. The principal roles of the intestinal bacterial community, as reported in terrestrial species, are to promote nutrient supply, prevent pathogen colonization and to maintain energy homeostasis and mucosal immunity (Gill et al 2006, Oakley et al 2014, Yeoman & White 2014). Comparatively little is known of the composition and specific functions of these communities in the fish GI tract (Nayak 2010). The elevated presence of bacterial species in the fish gut, when compared with the microbial assemblage in the surrounding aquatic environment, may suggest that the intestine provides a unique ecological niche for a diverse but select group of microbes (Cahill 1990, Ringø et al 1995, Nayak 2010), however this is still the subject of debate. Bacteria are thought to be present in this ecosystem as both transient (allochthonous) and resident (autochthonous) populations. The autochthonous microbes colonize the intestinal wall, whilst the allochthonous bacteria are most often located in the intestinal lumen (Ringø et al 2001). Some of these transient bacteria may also have the ability to colonize the intestinal wall, especially if the mucosal layer of the intestinal epithelium is compromised (Hansen & Olafsen 1999, Dimitroglou 2009).

The fish GI tract is inundated with bacteria from a wide range of sources from the natural environment. Bacteria begin to enter the fish digestive system at the mouth opening stage of larval development and may colonize the developing digestive tract to become the early autochthonous microflora of juvenile fish (Hansen & Olafsen 1999, Cahill et al 1990, Ringø et al 1995, Nayak 2010). Bacteria that colonize the surface of fish eggs and those that are present in hatchery feeds may also play a role in the development of the early gut microflora (Ringø & Birbeck 1999, Romero & Navarrete 2006). In order to survive and proliferate in the gut, both autochthonous and allochthonous microbes must be able to resist the action of digestive enzymes and tolerate fluctuations in pH, especially when passing through the fish stomach, where the pH is 2-4 (Dimitroglou et al 2011). In addition to this, the bacterial species composition may be sensitive to a number of factors affecting the fish host, including changing

environmental conditions (Yoshimizu & Kimura 1976, MacFarlane et al 1986, Hagi et al 2004), developmental stage (Verner-Jeffreys et al 2003, Romero & Navarrete 2006), digestive physiology (Cahill 1990), and feeding strategy (Holben et al 2002, Uchii et al 2006, Dimitroglou et al 2011). Collectively, these microorganisms therefore make up the 'normal' microbiome of the fish intestine, which has been defined as *'the community of microbes present in most individuals of a population or a species that, despite continual contact with different tissues, causes no harm to the host'* (Romero et al 2014).

Once the gut microbial communities have established stable populations, they can interact with the host and may affect nutrition, growth, reproduction, overall population dynamics, and vulnerability to disease (MacFarlane et al 1986). *Bacteroides thetaiotaomicron*, a prominent member of healthy murine and human intestinal microflora, can modulate the expression of genes involved in several important intestinal functions, including nutrient absorption and mucosal barrier fortification (Hooper et al 2001, Stappenbeck et al 2002). Ringø et al (1995) suggested that *Bacteroides* spp. and *Clostridium* spp., organisms closely related to *B. thetaiotaomicron*, can enhance the nutritive capacity of fish through the synthesis of vitamins and essential fatty acids. Members of the phylum Firmicutes, especially the lactic acid bacteria (LAB), have also been suggested as having the potential to beneficially affect host health and nutrition, principally in terms of pathogen exclusion and fatty acid metabolism (Raida et al 2003, Carmody & Turnbaugh 2012, Semova et al 2012). Due to these factors, some of these bacterial groups have been promoted as potential probiotic organisms. Indeed, several studies have found that including these microbes as probiotic components in the diets of farmed fish can benefit fish health in terms of their intestinal efficiency and consequently their growth rate (Irianto & Austin 2002, Burr et al 2005, Merrifield et al 2010a, b).

The structure of the mammalian gut microbiome has been shown to group according to digestive physiology, with hindgut fermenters, foregut fermenters and those with simple gut structures with minimal differentiation each hosting different gut microbial communities (Muegge et al 2011). It is possible that the fish gut microbiome is also influenced by digestive physiology. However, fish GI bacterial communities are considered to be much less diverse than those of mammals (Trust et al 1979, Sakata 1990, Holben et al 2002), with an estimated 10^6 to 10^8 colony forming units (CFU)/g^{-1} tissue present within the fish intestine at the adult stage of development (Kim et al 2007). This is in comparison to ~10^{11} CFU/g^{-1} tissue present in the gut of terrestrial animals and humans (Moore & Holdeman 1974, Mead 1997). A recent study of gut microflora from humans and 59 other mammals found a strong correlation between

gut physiology and microbial community composition (Ley et al 2008), with bacteria of the phyla Firmicutes and Bacteroidetes dominating the gut microflora. It could therefore be possible that the same may be true of fish intestinal micro-communities, in spite of their reported lower levels of microbial diversity.

Recent advances in DNA sequencing technologies have enabled researchers to study the microbial communities present in the GI tract of animals at an unprecedented level of detail, to the extent that some now regard these micro-communities as a 'new organ' that may actively contribute to host metabolism (O'Hara & Shanahan 2006). To date however, these new technologies have rarely been used in studies of the fish gut microbiome. Therefore, the application of 'next generation sequencing' (NGS) technologies in investigating possible links between abundances of intestinal microbial taxa, and phenotypic and physiological states in fish, may pave the way for improved nutritional strategies and therefore improved fish health and production. It is therefore essential that the power of these modern molecular technologies is harnessed to produce a comprehensive and detailed analysis of the fish intestinal microbiome, in order to better understand the dynamics and possible effects of this microbial ecosystem on fish health.

1.3.2. Current knowledge on microbial composition

The early studies of the teleost fish gut microbiota involved the analysis of homogenates of intestinal content and/or faecal material using bacterial culture-based techniques on selective or general purpose media, followed by phenotypic characterization using conventional morphological and biochemical assays (Nayak 2010). The earliest published investigations of the fish gut microflora were conducted by Reed & Spence (1929) and MacFarlane-Stewart (1932). These studies examined the intestinal microflora of haddock (*Gadus aeglefinus*) and successfully cultured a variety of organisms, identifying isolates of *Proteus, Pseudomonas*, *Achromobacter* and spore-forming *Bacillus* as the dominant bacteria present in the digestive system of this species. A number of further initial studies used microscopic examinations and culture-based methods to characterize the intestinal microflora of a variety of other fish species including jack mackerel (*Trachurus japonicas*) (Aiso et al 1968), chum salmon (*Oncorhynchus keta*), steelhead trout (*Salmo gairdneri*), coho salmon (*Oncorhynchus kisutch*), pink salmon (*Oncorhynchus gorbuscha*), sockeye salmon (*Oncorhynchus nerka*) king salmon (*Oncorhynchus tshawytscha*) (Seki 1969, Yoshimizu & Kimura 1976) and yellowtail (*Seriola*

quinqueradiata) (Sakata et al 1978) noting an assortment of genera within the phylum Proteobacteria as dominant in all of the cultures analysed.

Liston (1957) was among the first scientists to posit that host-specific pressures select for a structured indigenous microflora in teleost fish and the first tangible evidence for this theory arrived with the study of Fishelson et al (1985), who demonstrated that *Epulopiscium* bacterial symbionts of tropical herbivorous surgeonfish were not found outside of the host. The work of Fishelson et al (1985) ignited an enhanced research effort into the area of the fish gut microflora. In the immediate aftermath of the Fischelson study, Lindsay & Gooday (1985) reported similar findings in that the intestinal microflora of cod (*Gadhus morhua*) was specialized and did not reflect organisms detected in the surrounding environment. Further characterization work continued with bacteria cultured from gut homogenates of a variety of fish species (Sugita et al 1983, 1985, Sakata 1990). This research effort culminated in reviews by Cahill (1990) and Ringø et al (1995) who concluded that fish, like other vertebrates, harbour specialized gastrointestinal communities. Thereafter the use of molecular methods as a tool to complement the findings of classical microbiological techniques became increasingly prevalent in studies of the fish gut microflora. These methods have since revealed that the level of recovery on traditional growth media of bacterial communities from the intestine of many fish species can be as low as <1% (Romero & Navarrete 2006, Navarrete et al 2009, Aguilera et al 2013).

Proteobacteria and Firmicutes are the most frequently reported phyla in the salmonid gut microbiota, suggesting that members of these phyla are especially well adapted to the intestinal ecosystem of these fish species. Other phyla that have commonly been reported are the Actinobacteria, Bacteroidetes, Fusobacteria, Tenericutes and Deinococcus-Thermus (Fig. 1.6). A recent study using pyrosequencing technology reported the presence of a total of 13 bacterial phyla in the rainbow trout intestine (Lowrey et al 2015). Much of the research thus far has however reported that the ecological evenness in the intestine is often skewed in favour of one or more microbial communities. For example, Holben et al (2002) reported that *Mycoplasma* represented 81% and 96% of the total microbial community recorded in the intestines of farmed and wild Atlantic salmon respectively. Pond et al (2006) used a molecular cloning approach to describe the microbial flora of farmed rainbow trout (*Oncorhynchus mykiss*), and reported that clone libraries were dominated by two major groups, *Aeromonas* and *Clostridium*. Similarly, Kim et al (2007), using denaturing gradient gel electrophoresis (DGGE) analysis, also found that *Clostridium* dominated the gut microbiota of rainbow trout. A comparable pattern of

community dominance was reported by Navarrete et al (2009), in that *Pseudomonas* comprised greater than 60% of the intestinal microbial community of juvenile Atlantic salmon *Salmo salar*. It is unclear as to why such a pattern of low microbial diversity has been reported in the gut of salmonids. Ley et al (2008) reported that the gut microflora of mammals progressively increases in its diversity from carnivorous to omnivorous to herbivorous lifestyles, and so the same may hold true in carnivorous salmonid fish, although much more detailed research is required to explore this possibility further.

Bacterial communities within the GI tract of fish have been observed to differ between fresh and salt water species, with a predominance of Gram-negative bacteria over Gram positive bacteria in the intestine of several fish species (Sakata et al 1984, Ringø 1993, Hatha et al 2000). *Aeromonas* spp. have been reported to be the most common finding within the GI tract of freshwater fish (Sugita et al 1983, 1994, Wang et al 1994, Asfie et al 2003), whilst *Pseudomonas*, *Plesiomonas*, *Enterobacteriaceae*, *Micrococcus*, *Acinetobacter*, *Carnobacterium* and *Clostridium* have also been documented (Sugita et al. 1985, Cahill 1990, Ringø et al 1995, Kim et al 2007). In contrast to freshwater fish, *Vibrio*, *Pseudomonas*, *Achromobacter*, *Corynebacterium*, *Alteromonas*, *Flavobacterium* and *Micrococcus* species have been found to dominate the GI tract of most of the marine fish species (Cahill 1990, Ringø et al 1995, Verner-Jeffreys et al 2003, Nayak 2010).

LAB are also commonly present in the GI tract of both freshwater and marine fish species, however, they are not recorded as regularly as many of the Gram-negative bacterial species. There is an extensive body of research on LAB in the gut of endothermic animals, as these bacteria have the ability to successfully colonize the gut, and they are antagonistic against Gram-negative pathogens (Goldin 1986, Conway 1989, Sissons 1989, Gorbach 1990, Goldin & Gorbach 1992, Jonsson & Conway 1992). Therefore, LAB could possibly have a role in promoting host nutrition and are often fed to these animals as probiotic supplements. There has been comparatively little research carried out into the role of LAB in fish. Ringø & Gatesoupe (1998) reviewed the subject, and identified *Streptococcus*, *Leuconstoc*, *Pediococcus*, *Aerococcus*, *Enterococcus*, *Vagococcus*, *Lactobacillus*, *Lactococcus* and *Carnobacterium* as the main LAB that colonize the GI tract of fish. Kim & Austin (2006) examined the potential of *Carnobacterium* to inhibit the colonization of Gram-negative fish pathogens in the intestine of rainbow trout. The study found that this bacterium produced a broad spectrum of inhibitory effects against potentially pathogenic bacteria. More recent research, using high throughput sequencing technologies, has found that LAB represent a minor portion of the total microbial

diversity in the fish GI tract (Desai et al 2012, Wong et al 2013) and so further research is required to validate these findings and to examine potential methods of fortifying and enriching these communities to promote the digestive health of rainbow trout.
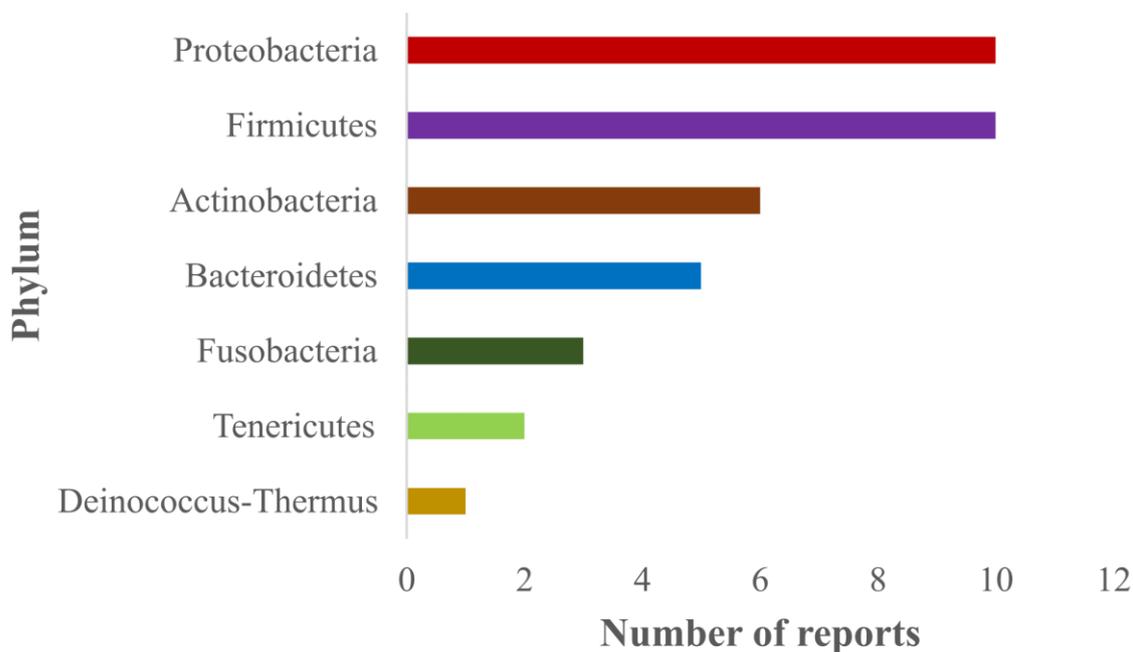
Figure 1.6. Bacterial phyla reported in the intestine of salmonids (adapted from Nayak 2010).

1.3.3. Host factors and the core microbiome concept

The intimate relationship between aquatic animals and specific microbial populations has been well described for a number of different species of sponges, shrimps and finfish (Griffiths et al 2001, Hentschel et al 2002, Lau et al 2002, Holben et al 2002, Rawls et al 2004, Mansfield et al 2010). Perhaps the most spectacular exhibition of this relationship is that of the Hawaiian bobtail squid (*Euprymna scolopes*) and its luminescent Gram-negative bacterial symbiont *Aliivibrio fischeri* (McFall-Ngai 2014). The presence of several distinct bacterial classes, in the intestinal microbiome of the same fish species from different populations and geographic locations, indicates that these bacteria may be important contributors to host gut functions and that they have possibly co-evolved with their fish hosts. This concept is often referred to as the 'core gut microbiota' hypothesis. This theory has mainly been explored in mammalian hosts, but is beginning to be explored in fish (Rawls et al 2004, Roeselers et al 2011, van Kessel et al 2011, Desai et al 2012, Wong et al 2013, Givens et al 2014, Ingerslev et al 2014). The core gut

microbiome may be shaped by host intestinal physiology, immunity or other related factors and is deserving of further research, especially in commercially important farmed fish species.

The natural bacterial flora from the surrounding aquatic environment has been proposed to be a principle source of the microbial colonization of the GI tract in farmed fish. Wu et al (2012, 2013) reported that pond water and pond sediment provided seed populations of microbes, which were then detected in the gut of both cultured grass carp (*Ctenopharyngodon idella*) and gibel carp (*Carassius gibelio*). Similarly, Semova et al (2012) found that many of the bacterial taxa recorded in zebrafish intestines were also present in similar abundances in their tank water, but importantly they also noted that some assemblages appeared to be much more abundant in the GI tract of these fish. Sullam et al (2012) performed a meta-analysis of previously sequenced libraries of the gut microbiota of freshwater and marine fish. They found an increased representation of *Aeromonas* in the freshwater fish and a dominance of *Vibrio* in the marine species, reflecting many of the findings of earlier studies of the fish gut microflora. Xing et al (2013) characterized the gut microbiota of farmed adult turbot (*Scopthalmus maximus*) and reported that the GI tract of these fish consisted of bacteria initially associated with seawater. Collectively, these findings suggest that the fish gut may closely resemble the surrounding aquatic environment, indicating a minimal role of selective pressures imposed by the host in shaping these microbial communities.

In contrast to this, other studies have reported that the structure of the fish intestinal microbiome is not a simple reflection of the bacteria present in the surrounding environment. These studies have described distinct gut microbial community compositions that are different to those of the aquatic ecosystems that they inhabit. Roeselers et al (2011) found that both wild and domesticated zebrafish shared a common 'core' microbiome independent of life history and geographical location. Another study on the juvenile paddlefish (*Polyodon spathala*) and bighead carp (*Hypothalmichthys nobilis*) reported a species specific core microbiome structure, even though both species were raised in the same pond with access to the same food sources (Li et al 2014). Similarly, Li et al (2015) analyzed the gut microbiome of three separate species of carp raised in the same environment, and they found that each species harboured a unique intestinal microbiome. Furthermore, Navarrete et al (2012) reported slight differences in the composition of the intestinal microbiome of rainbow trout that shared the same rearing facility but originated from different genetic stocks. More recently, Llewellyn et al (2015) reported that Atlantic salmon, obtained from separate geographical locations, shared a common core microbiome that was significantly different to that of the surrounding environment. The authors

suggested that life stage, rather than environment or geographical location, was a stronger predictor of the structure of the intestinal microbiome.

It is therefore clear that further investigation is required to determine the potential presence of a core intestinal microbiome in rainbow trout and the host factors that govern its composition. Information on the core taxa that inhabit the intestine could improve our understanding of normal microbial populations in healthy fish. This knowledge could potentially lead to an ability to recognize imbalances in the rainbow trout intestinal microbiome (e.g. potentially caused by stress, antibiotic therapy etc.), and therefore could result in the development of safe and effective methods of altering the composition of the microbiome in order to restore microbial homeostasis (Turnbaugh & Gordon 2009, Roeselers et al 2011, van Kessel et al 2011, Wu et al 2012, Ghanbari et al 2015). Through careful modulation of the core intestinal microbiome it may be possible to improve bacterial metabolite production, stimulate immune signalling pathways and strengthen host defence mechanisms, but detailed data on the microbial ecology of the intestine must first be collected.

1.3.4. The influence of diet on the intestinal microbiome

A large body of research using diverse vertebrate models, including broilers, swine, mice, humans and to a lesser extent fish, have demonstrated that dietary strategies can alter the structure of the intestinal microbiome (Turnbaugh et al 2006, 2009, Turnbaugh & Gordon 2009, Kostic et al 2013, Upadrasta et al 2013, Romero et al 2014). It has been suggested that the fermentation of dietary components by members of the intestinal microbiome results in the production of a large variety of metabolites, which may have beneficial effects on the health of the host. In humans, this area has achieved particular attention due to the possible links between digestive and metabolic disorders such as irritable bowel syndrome (IBS) and Crohn's disease, and the intestinal microbiome. There is now good evidence to suggest that diet plays a role in the development of these diseases, as most patients have reported that their symptoms can be triggered by specific foods. This observation, coupled to a perceived microbiome dysbiosis in this condition, is suggestive of an interrelationship between diet and the regulation of the structure of the intestinal microbiome in the manifestation of this disease (Lewis et al 2015, Øyri et al 2015). Furthermore, specific members of the gut microbiota have been observed to be positively correlated with obesity in humans and mice (Turnbaugh et al 2006, Ley et al 2008). Obese mice and humans have been observed to possess altered Firmicutes:Bacteroidetes ratios compared with lean individuals, and exhibit an accompanying decrease in bacterial

diversity. Whilst obesity is undesirable in humans, the potential to manipulate specific members of the intestinal microbiome in order to increase weight gain in cultured fish species is an attractive possibility.

The possible link between dysbiosis in the intestinal microbiome and digestive disorders has received comparatively little attention in cultured aquatic species. This is in some part due to the fact that so little is known about the natural composition of the intestinal microbiome in fish. Some research has linked enteritis, observed in Atlantic salmon fed soy bean meal, to a dysbiosis in the intestinal microbiome (Merrifield et al 2011, Desai et al 2012, Green et al 2013), but this finding must be viewed with caution considering that the structure of the intestinal microbiome in healthy fish has not yet been fully revealed. The investigation of the relationship between diet and the intestinal microbiome in fish may also improve our understanding of the role of these microorganisms in the metabolism of different dietary ingredients in higher vertebrates. Furthermore, recent shortages in fish meal and fish oil sourced from wild capture fisheries, coupled with the drastic reduction in antibiotic use in aquafeeds has precipitated the development of a variety of 'functional feeds'. Functional feeds can be used to replace components of the diet, or fed as supplements. The goal of these diets is to improve the health of farmed fish by enhancing immunity, growth, and performance, through supplying additional compounds above the basic level of nutritional requirements necessary for fish growth. However, very few studies have tested the health effects of these functional feeds on commercially important farmed fish (Martin et al 2003, Frøystad et al 2008, Leaver et al 2008), and an understanding of their potential impact on the intestinal microbiome requires that detailed data is generated on the baseline composition of these communities in different species. The majority of these functional feeds can be divided into three main categories: probiotics, prebiotics and synbiotics.

1.3.4.1. Probiotics

Merrifield et al (2010a) defined the term 'probiotic' as *'any microbial cell provided via the diet or rearing water that benefits the host fish, fish farmer or consumer, which is achieved, in part at least, by improving the microbial balance of the fish'*. In an aquaculture context, host benefits are considered to be an elevation in disease resistance, immunomodulation and improved intestinal morphology. Benefits to the fish farmer are considered to be improved fish appetite, growth performance, feed conversion ratio (FCR), flesh quality and reduced malformations (Lauzon et al 2014). It must be stressed that the concept of 'microbial balance' is still poorly

understood in fish, and so the evaluation of any potential health benefits of probiotics requires a more detailed assessment of the true microbial diversity of the fish intestine. A range of criteria must be met before a specific bacterial strain can be classed as having probiotic properties. The most important of these criteria is that the probiotic must; 1) originate in the host fish, 2) be able to survive and multiply in the GI tract, 3) be resistant to bile acids, 4) lack any plasmid-encoded antibiotic resistance genes, and 5) have the capacity to confer clear health benefits to the host fish through the modulation of the intestinal microbiome (Lauzon et al 2014). There are a number of mechanisms by which probiotic bacteria can improve the health of farmed fish. For example, they may inhibit the adhesion of pathogens to the intestinal epithelium, increase the production of goblet cells, mucous and bacteriocins, modify the immune system through enhanced cell-mediated responses, and contribute to digestive processes through the production of extracellular digestive enzymes (Merrifield et al 2010a, b, Montalban-Arques et al 2015).

The majority of probiotic organisms tested in fish to date have been members of the LAB. LAB are Gram-positive, often non-motile, catalase- and oxidase-negative, non-spore forming bacteria, and they produce lactic acid as a major end product of their fermentative metabolism. They are resistant to bile acids and pancreatic enzymes, and are therefore often reported to be inhabitants of the nutrient-rich intestines of endothermic and ectothermic animals. *Lactobacillus* and *Carnobacterium* represent some of the most extensively studied genera with potential probiotic capabilities in salmonids. Studies that have successfully altered the gastrointestinal microbiota of fish with *Lactobacillus* strains have shown that these changes have led to improvements in fish health in terms of immune status, growth performance and stress response (Merrifield et al 2010a, Dimitroglou et al 2011, Merrifield & Carnevali 2014).

Carnobacterium species have been widely reported to multiply and compete with indigenous microbes in the GI tract of salmonids (Merrifield & Carnevali 2014). *Carnobacterium divergens* strains appear to be particularly effective in cold water species, however in warm water species the growth optima of these microbes suggests that they may be more easily outcompeted by other indigenous members of the microbiome. *Lactococcus*, *Streptococcus*, *Enterococcus*, *Bacillus*, *Pediococcus* and *Leuconostoc* have also been shown to exert probiotic effects in the GI tract of salmonids (Merrifield et al 2014). Whilst earlier culture dependent studies suggested that LAB were dominant members of the intestinal microbiota of salmonids, culture independent methods have shown that this may not be the case. Desai et al (2012) reported that many of these genera are present in abundances of ~1% in the rainbow trout

intestine, and in some cases, such as with *Enterococcus*, can represent <0.1% of the total microbial population. However, although it is possible that the LAB are not generally the dominant group among the rainbow trout intestinal microbiome, the prevalence and role of these bacteria is deserving of further investigation, as their functional activity may offer distinct health benefits. Therefore, further research is required to assess the potential probiotic or prebiotic methods of boosting these microbial populations within the rainbow trout intestine.

1.3.4.2. Prebiotics

Prebiotics can be defined as dietary ingredients that are selectively fermented by specific intestinal bacteria, which result in changes in both the composition and activity of the microbiome (Gibson & Roberfroid 1995, Gibson et al 2004). These changes increase the release of microbial metabolites that improve feed digestion, are beneficial to intestinal epithelial cells, modulate the immune system, and can inhibit the growth of potentially pathogenic bacteria. A range of prebiotics have been tested in salmonid aquaculture. The most promising of these have been reported to be inulin, fructooligosaccharides (FOS), mannanoligosaccharides (MOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS) and arabinoxylooligosaccharides (AXOS). These prebiotics are mainly composed of poorly digestible carbohydrates with a relatively short chain-length, classified on the basis of their molecular weight. Carnivorous fish, such as the salmonids, have difficulty digesting carbohydrates that are not present in their natural diet, however the above mentioned prebiotics have been shown to confer numerous health benefits to these fish (Burr et al 2005, Gatlin et al 2006, Denev et al 2009, Merrifield et al 2010b, Ringø et al 2010, Sweetman et al 2010 and Dimitroglou et al 2011).

It is known from studies in herbivorous and omnivorous fish species that the gut microbiota plays a role in fermenting indigestible feed ingredients, usually carbohydrates, and produces short chain fatty acids (SCFA) as the end product of this action (Stevens & Hume 1998, Clements et al 2009). Some authors have claimed that the level of SCFA in the gut of fish may reflect the total contribution of the intestinal microbiome to the overall energy requirements of the host fish. For example, the gut of herbivorous fish has been found to contain high levels of SCFA, in combination with an enrichment in members of the phylum Firmicutes, which suggests that this bacterial phylum may play a key role in the fermentation of indigestible plant carboydrates, and in the subsequent production of beneficial metabolites that can be utilized as an energy source (Mountfort et al 2002, Moran et al 2005, Skea et al 2005, 2007, Clements et

al 2007). Thus, the indigestible carbohydrate fraction of the diet of these fish exerts a prebiotic effect in this case. Clements et al (2014) argued that the intestinal microbiome is likely to be of more importance to the digestive processes in herbivorous fish that ingest large amounts of carbohydrate as part of their plant and algae-rich diets. However, Smith et al (1996) reported that carnivorous fish intestines can contain relatively high levels of SCFA, suggesting that microbial fermentation may also be important in the digestive physiology of these fish. A recent study by Asakura et al (2014) found that the metabolite profiles and the structure of the intestinal microbiome in carnivorous and herbivorous fish clustered according to feed type when cultured under controlled conditions. The results of this study, which used an NGS based approach to profile the intestinal microbiome, illustrate the potential utility of prebiotics in altering these communities in farmed fish species, in order to exploit their digestive capacity. Carnivorous fish derive the majority of their energy from dietary protein, and so the quantitative contribution of the distal intestinal microbiome to protein digestion represents another potentially interesting avenue of investigation.

The enrichment of beneficial members of the intestinal microbiome of fish with the use of prebiotics could be a promising method for improving their health. The fermentation of prebiotic carbohydrates, and possibly proteins, that escape digestion in the anterior intestine, can lead to the production of a variety of microbial metabolites such as SCFA, which are a primary energy source for the intestinal epithelial cells (Hamer et al 2008). Therefore, by accurately characterizing the intestinal microbiome of rainbow trout and by assessing how these communities respond to different dietary ingredients, it may be possible to reveal those microbes that play central roles in the production of metabolites as a result of the selective fermentation of dietary compounds, which the host cannot degrade on its own. This knowledge could be of great importance when designing new aquaculture feeds that aim to increase nutritional gain from feed components, through harnessing the metabolic potential of these complex microbial communities.

1.3.4.3. Synbiotics

Synbiotics combine both prebiotics and probiotics in a dietary supplement. A selected probiotic or combination of probiotics is fed to the animal at an early stage of development, before the subsequent addition of a prebiotic. The prebiotic consists of a substrate that is preferentially metabolized by the supplemented probiotic bacterial species, and hence helps to establish the introduced bacteria in the intestine and to harness their beneficial effects on intestinal health.

This approach has been reported to be effective in modulating the intestinal microbiome of the domestic chicken (*Gallus gallus domesticus*). It has also been referred to as the 'Seed, Feed, Weed' method of managing the intestinal health of these animals (Oakley et al 2014, Mundt et al 2015).

A limited number of synbiotic trials have been carried out on farmed fish species. Haghighi et al (2010), Firouzbakhsh et al (2012) and Nekoubin et al (2012) all reported improvements in growth, FCR, specific growth rate (SGR) and survival in kutum (*Rutilus kutum*), rainbow trout and zebrafish (*Danio rerio*) respectively, when fed a commercial synbiotic, Biomin IMBO™. This synbiotic product contains a combination of a proprietary strain of *Enterococcus faecium* and FOS. Tapia-Panigua et al (2011) reported a decrease in microbial diversity and greater microbial similarity indices in the intestine of gilthead sea bream (*Spaurus auratus*) fed a synbiotic containing a combination of the yeast *Debaryomyces hansenii* in combination with inulin after four weeks of feeding. These authors also detailed the up-regulation of a suite of genes regulating intestinal immunity after two weeks of supplementation with the synbiotic.

Abid et al (2013) described the modulation of the gut microbiota of Atlantic salmon fed a combination of the probiotic bacteria *Pediococcus acidilactici* and prebiotic short-chain FOS after a 63-day feeding trial. In this study, the population of the beneficial *P. acidilactici* was found to have markedly increased by the end of the experiment, indicating that it was firmly established in the intestine of the test fish. Two recent studies by Cerezuela et al (2013a, b) showed that a combination of *Bacillus subtilis* and inulin produced mixed results when fed to gilthead sea bream. Synbiotic supplementation resulted in significantly increased intestinal villi height, but a decrease in the number of goblet cells and microvilli height when compared to a control group fed a standard commercial diet. Furthermore, a significantly lower microbial richness was recorded in the synbiotic fed fish, and a greater level of mortality was noted in synbiotic fed fish challenged with the pathogenic bacteria *Photobacterium damselae* subsp. *piscicida*. Therefore, synbiotic feeding strategies could undoubtedly be effective in aquaculture, but further research is warranted to determine in more detail both the response of the indigenous gut microbiome to such dietary ingredients, and the optimal inclusion rates needed to produce tangible benefits to fish health.

1.3.5. Potential function of the fish intestinal microbiome

It is generally believed that the intestinal microbiome influences various host functions including development, digestion, nutrition, disease resistance and immunity at both a localized and systemic level. The role of the intestinal microbiome in nutrition and metabolism is perhaps the best studied. Recent studies have suggested that the cellulolytic microbes present in the gut of cyprinid fish can contribute to the digestion of plant material within the intestinal tract of these fish (Wu et al 2012, Ni et al 2014). Similar inferences of microbial cellulolytic activity in the intestine of fish have been made for wood-eating loricariid catfishes such as *Panaque nigrolineatus* (Nelson et al 1999, Nonogaki et al 2007, Di Maiuta et al 2013). Some herbivorous marine fish have been reported to be reliant on the fermentative action of their intestinal microflora in assimilating indigestible algal components such as mannitol. The end products of such fermentations are SCFA, such as butyrate, acetate and propionate, which may be metabolized by these fish as an energy source and which have additionally been shown to improve intestinal epithelial cell function in other animals (Hamer et al 2008). Thus, the concentration of SCFA in the distal intestine has been highlighted as potentially being indicative of the relative contribution of the intestinal microbiome to the digestive processes of fish. Whilst the Clostridia have been suggested as being responsible for the production of SCFA in the fish intestine (Clements et al 2007), further work is required to fully characterize the range of potentially fermentative microorganisms present.

The intestinal microflora of fish may also produce a range of enzymes that complement the endogenous digestive enzymes produced by the host to aid in carbohydrate and protein metabolism. Ray et al (2012) reported the presence of amylase, protease, lipase, chitinase, cellulase and phytase-producing bacteria isolated from the digestive tract of a variety of fish, and suggested that these bacteria could contribute to the enzymatic breakdown of dietary ingredients. Similarly, Smriga et al (2010) suggested that members of the Proteobacteria, Bacteroidetes, Firmicutes and Fusobacteria may all contribute to the digestion of cellulose-rich material in tropical reef fish through the production of a variety of enzymes. The intestinal microbiome of fish can also synthesize vitamins (Roeselers et al 2011). A number of recent studies have reported the presence of *Cetobacterium somerae*, a potent vitamin B12-producing Gram-negative bacteria, in the intestinal tract of fish (Tsuchiya et al 2008, Larsen et al 2014, Baldo et al 2015). A correlation has been drawn between the presence of this microbe in the GI tract and the dietary requirement of vitamin B12 for individual fish species. For example, *C. somerae* has often been reported in tilapia and carp, species that have no vitamin B12

requirement. In contrast, this microbe is not a common component of the microflora of Japanese eel and channel catfish, species that have a dietary requirement for this vitamin (Romero et al 2014). Further research is however required in order to quantify the contribution of the entire intestinal microbiome to vitamin production.

The relative contribution of the intestinal microbiome to digestive physiology in fish has been hypothesized to be linked with the morphology of the GI tract of different species (Ley et al 2008). For example, herbivorous and omnivorous fish tend to have long digestive tracts that are adapted to the breakdown of indigestible, mainly plant-based, dietary ingredients. The residence time of the ingested material is thus longer in these fish, which possibly presents more of an opportunity for microbial fermentation to occur in the intestine. Therefore, it has been suggested that these species, many of which do not possess a stomach, may be more reliant on the relative contribution of the intestinal microbiome to their digestive processes. In contrast, it has been posited that carnivorous fish, with short gut transit times and high protein diets, may rely more heavily on the contribution of endogenous digestive enzymes. However, carnivorous fish have been shown to produce relatively high concentrations of intestinal SCFA (Smith et al 1996), suggesting that comparable levels of gut fermentation may occur in both herbivorous and carnivorous fish species. Furthermore, fish differ from many terrestrial animals in terms of their mechanisms of protein uptake and intestinal microbiome composition, suggesting that the microbiome of the distal intestine may also make a significant contribution to host protein metabolism (Clements et al 2009, 2014).

Studies by Rawls et al (2004, 2006) have shown that the GI microbiota of zebrafish can regulate the expression of a total of 212 genes in the intestinal tract. Some of the genes recorded function in epithelial cell proliferation, innate immunity, and nutrient metabolism pathways. It is clear from mammalian studies that the presence of commensal bacteria aid in the development of the innate and adaptive immune systems (Rakoff-Nahoum et al 2004, Kelly et al 2005, Mazmanian & Kasper 2006, O'Mahony et al 2008, Hooper et al 2012). The GI tract, in addition to the skin and gills, is thought to be a major route of entry for potentially pathogenic bacteria in fish, and the commensal intestinal microbiome can protect against pathogen colonization by either competitive exclusion, or via the production of toxic metabolites such as bacteriocins (Ringø et al 1995, Nayak 2010). Should a disturbance occur that causes an imbalance, or dysbiosis, in the commensal microbiome, the fish may be left vulnerable to pathogenic infection. Bacterial genera commonly found in the intestine of fish, including *Vibrio*, *Aeromonas*, *Flavobacterium*, *Edwardsiella*, *Pseudomonas*, *Photobacterium*, *Yersinia* and

Renibacterium all possess strains that are capable of causing disease (Llewellyn et al 2014). Moreover, many of these microbes often comprise minor components of the intestinal microbiome and only emerge as opportunists when the fish is stressed or when its immune system is compromised.

Much of the literature often refers to imbalances or perturbations in the fish intestinal microflora and the potential adverse effects that can arise as a result. However, very little is known about the normal composition of the intestinal microbiome in healthy rainbow trout. Much of what we do know about the make-up of these communities is based on the results of studies conducted with culture-based and low resolution molecular techniques. Therefore, much more detailed information is required in order to understand the structure of the rainbow trout intestinal microbiome in healthy and stressed/diseased states. This thesis will attempt to address this area by using high-throughput sequencing to build comprehensive taxonomic baselines of microbial taxa in healthy rainbow trout. These baselines can then be further used in conjunction with metagenomic approaches in order to enhance our understanding of the contribution of the intestinal microbiome to physiological processes in these fish.

1.4. Next generation sequencing (NGS) and the fish intestinal microbiome

1.4.1. The bacterial 16S rRNA gene

The microbiologist Carl Woese laid the foundations for the culture-independent characterization of microorganisms with his research on the molecular phylogeny of bacteria in the 1980's. At approximately the same time, the polymerase chain reaction (PCR) was being developed by Kary Mullis, and this provided the basis for Woese's studies (Woese 1987). Woese focused on the small subunit of bacterial ribosomes, the molecular machines responsible for microbial protein synthesis. Within this small subunit of the molecule are structural regions that are highly conserved due to the lethality of mutants and the lack of silent mutations that can occur in protein coding genes. However, some of the regions within this molecule are less important structurally and gradually accrue mutations at a phylogenetically useful rate. Therefore, Woese discovered that the PCR amplification and identification of the genetic sequence of these particular 'hypervariable' regions of the molecule could act as potent delineators of different bacterial groups.

The characterization of the hypervariable regions of the prokaryotic 16S rRNA gene now form the basis for the modern day culture-independent classification of bacterial species. The mature prokaryotic ribosome includes 16S (1541bp), 23S (2930bp) and 5S (190bp) molecules. Among these molecules, the 16S is the most studied due to its shorter length and hence ease of amplification. The 'S' in the notation refers to Svedberg units measuring centrifugation rate. The terms rDNA and rRNA are often used interchangeably in the microbial ecology literature. The term rDNA refers to the bacterial chromosomal DNA sequence from which the rRNA is transcribed. Current convention states that the term '16S rRNA gene' should be used (Claridge 2004). There are nine hypervariable regions of the 16S rRNA gene (V1-V9), and these are flanked by highly conserved regions (Fig. 1.7). These hypervariable regions demonstrate considerable sequence diversity among different bacteria and therefore represent attractive gene targets for PCR-based identification. Among these hypervariable regions, the V4 region has been highlighted as being particularly useful in classifying bacteria (Mizrahi-Man et al 2013).

The emergence of 16S rRNA based classification has vastly improved our understanding of bacterial taxonomy and diversity. Once amplified, 16S rRNA gene sequences are deposited in online databases, such as the ribosomal database project (RDP), Greengenes and SILVA, which can then be accessed by other researchers. The RDP is an open access annotated 16S rRNA gene database (http://rdp.cme.msu.edu/) that provides an invaluable tool for microbial ecologists in characterizing bacterial DNA directly from colonies, or from more complex environments and habitats, such as those of the soil and the GI tract of animals. The database is growing rapidly, particularly in light of the recent advances in high-throughput sequencing technologies and their use in characterizing previously unexplored microbial habitats and sample types. Consequently, a large proportion of the sequences in the databases are derived from uncultivable strains of bacteria, which highlights the advantages of targeting the 16S rRNA gene in culture independent studies. As of May 2015, in its most recently updated release, the RDP contained 3,224,600 16S rRNA sequences, more than doubling the number of sequences archived since August 2010 (1,418,497).
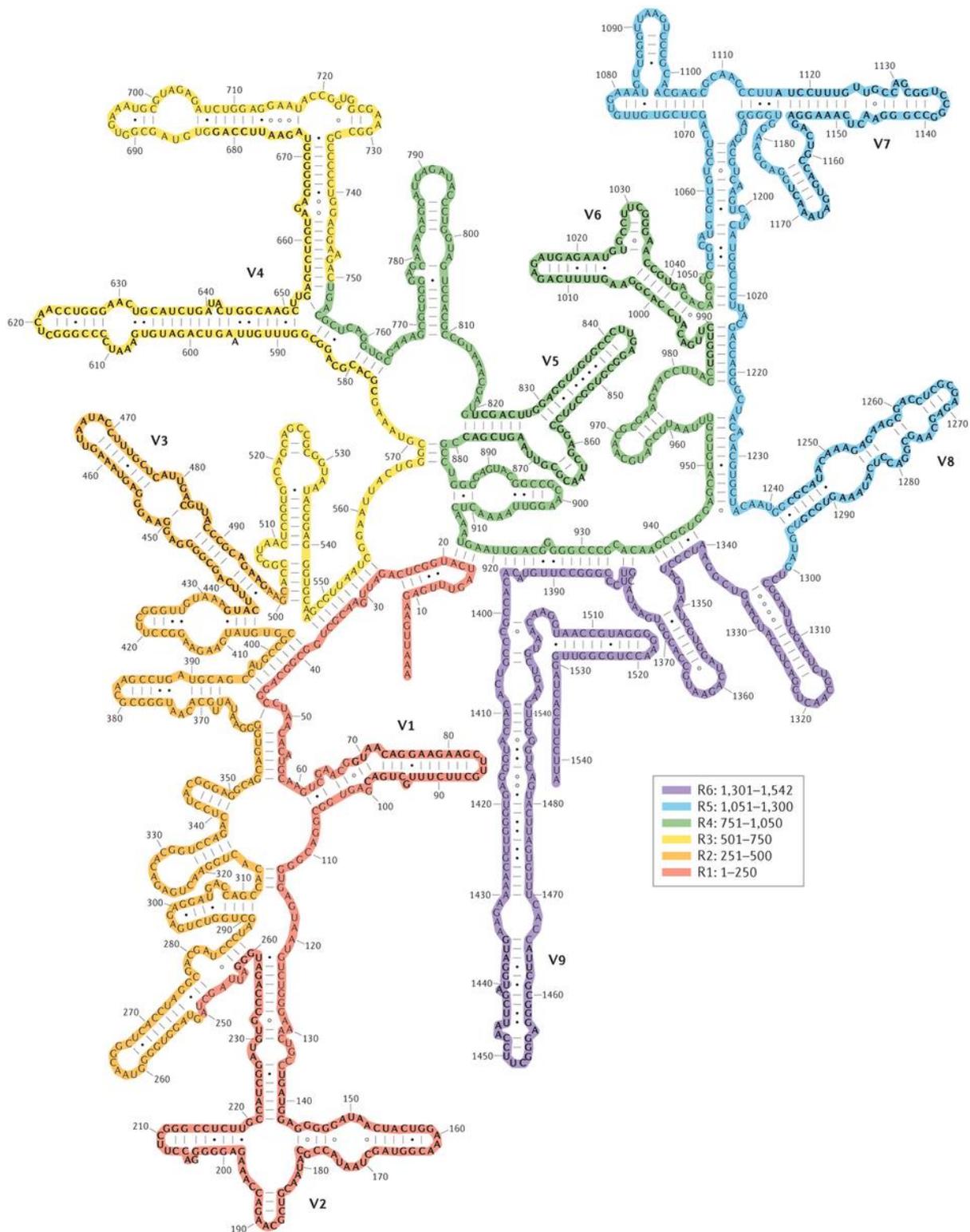
Nature Reviews | Microbiology

Figure 1.7. A diagrammatic representation of the 16S rRNA gene of *Escherichia coli*. The six main gene fragments are colour coded, and the hypervariable regions within each fragment are labelled (V1-V9) (from Yarza et al 2014).

1.4.2. NGS-based microbiome analysis

Low resolution molecular methods that target the 16S rRNA gene have presented researchers with a snapshot of the bacterial diversity present in the rainbow trout intestinal tract, however these techniques do not provide the coverage necessary to reveal the full array of microbes that inhabit this ecosystem. Less abundant or rare taxa, which are not captured by such techniques, may still play key roles in intestinal health and disease. The advent of NGS or **h**igh-**t**hroughput **s**equencing (HTS) technologies have presented researchers with a unique opportunity to gain new insights into the true microbial diversity present in the fish GI tract. These technologies permit massively parallel sequencing of 16S rRNA PCR amplicons, providing sufficient taxonomic resolution and coverage to capture a far greater proportion of the members of the intestinal microbiome, including rare bacterial taxa.

There are several NGS platforms currently available for the characterization of complex microbial environments, such as the Roche® GS-FLX 454, Ion Torrent®, Illumina® MiSeq, Illumina® HiSeq and PacBio® systems, and the suitability of each to the given aims of a microbiome sequencing project must be taken into careful consideration (Inglis et al 2012, Goodrich et al 2014, Ghanbari et al 2015). The two most important parameters to review, which impact upon phylogenetic resolution and coverage respectively, are the length of the sequence read generated and the number of sequenced reads produced. The Roche GS-FLX 454 platform has markedly improved in performance in recent years and can now provide an average read length of ~700bp using its FLX+ chemistry (Ghanbari et al 2015). The longer read length means that researchers can obtain a greater phylogenetic resolution, as the read can also span multiple hypervariable regions of the 16S rRNA gene. However, the GS-FLX 454 is more expensive than other platforms, and its total output of on average one million reads is a clear limitation for projects where coverage is prioritized over read length.

Long read lengths are preferable for 16S rRNA based characterization of microbial communities. However, short reads may provide comparable taxonomic resolution (Liu et al 2007, Luo et al 2012). For example, the V4 hypervariable region of the bacterial 16S rRNA gene is ~292bp long. Therefore, the entire region can be sequenced using the current Illumina sequencing chemistry, which offers 2 x 300bp paired end reads. Similarly, some researchers opt to use this read length to design primers targeting overlapping sections of different hypervariable regions, in order to improve taxonomic resolution. Further research is required to confirm whether this strategy has tangible advantages over amplicons produced from

primers aimed at a single region. The Illumina platforms are therefore advantageous both in terms of coverage and phylogenetic resolution, and given that many thousands of reads per sample are required to encompass the true bacterial diversity of the rainbow trout gut, they offer a powerful method of characterizing the intestinal microbiome of these fish.

The output of high-throughput surveys of the intestinal microbiome presents a bioinformatics challenge, in that millions of sequences must be analyzed and curated before taxonomic assignments can be made. Fortunately, a number of bioinformatics pipelines have been developed that enable the processing and interpretation of these enormous data sets. The most popular of these bioinformatics pipelines are mothur (Schloss 2009) and QIIME (Caporaso et al 2010). The output from a standard MiSeq run results in the production of two separate 'Fastq' files, one containing the forward reads of the hypervariable 16S rRNA region targeted and the other the corresponding reverse reads. The first step in the analysis is to assess the overall sequence quality using a quality control program such as FastQC (Andrews 2010). Thereafter reads can be trimmed as appropriate to ensure that they are of the same length. Trimming reads ensures that they merge successfully when analyzed in bioinformatics pipelines, and prevents sequencing errors and inflated estimates of bacterial diversity. Further tools within mothur or QIIME can remove sequences containing ambiguous base calls, homopolymers, chimeras and any other associated sequencing errors. Moreover, mitochondria and chloroplasts, thought to be the predecessors of bacteria and archaea, also contain 16S rRNA genes, which may be amplified in error, and can be removed from the dataset. The resulting quality controlled 16S rRNA sequences may be rapidly assigned to taxonomic ranks using the Bayesian classifier implemented by the Ribosomal Database Project (RDP). The relative abundance of each of the various taxa within a sample can thus be determined. Statistical analyses may also be performed using these pipelines, which can generate data on sequencing coverage in addition to important biological information on metrics such as community structure, diversity and richness.

1.4.3. Illumina high-throughput sequencing platforms

The Illumina high-throughput sequencing platforms were introduced in 2006, and they were adopted by many researchers as an attractive alternative to the more expensive 454-based pyrosequencing technology. At that time, 454-based pyrosequencing technologies could generate read lengths of ~450bp in comparison to ~100bp reads from Illumina. However, the Illumina platform could generate more data at a lower cost. Today's Illumina platforms are vastly improved and the MiSeq can now produce up to 25 million paired end reads of ~300bp

in length from a single run, thereby covering entire hypervariable regions of the 16S rRNA gene. The first step in sample preparation is to extract DNA from the environmental sample. Next, a PCR is carried out to amplify a chosen hypervariable region of the 16S rRNA gene. A second PCR is then performed that adds sequencing adapters and unique sample indices to the amplified fragments from each sample, which can then be pooled to create a library of amplicons (Figure 1.8). This library is then processed on the MiSeq using sequencing by synthesis (SBS) chemistry, which relies on the incorporation of dye terminator nucleotides into the sequence by a DNA polymerase (Siqueira et al 2012, Ghanbari et al 2015). DNA fragments are firstly immobilized on a flow cell surface, that is coated with adapters and complementary adapters. Each of these single DNA fragments creates a bridge with the complementary adapters. A reaction mixture containing DNA polymerase, sequencing primers and four reversible terminator nucleotides, each labelled with a fluorescent dye, are then passed over the flow cell. After incorporation, the terminator nucleotide and its position on the flow cell are detected and recorded by a four channel fluorescence scanner (Metzker 2010, Ghanbari et al 2015).
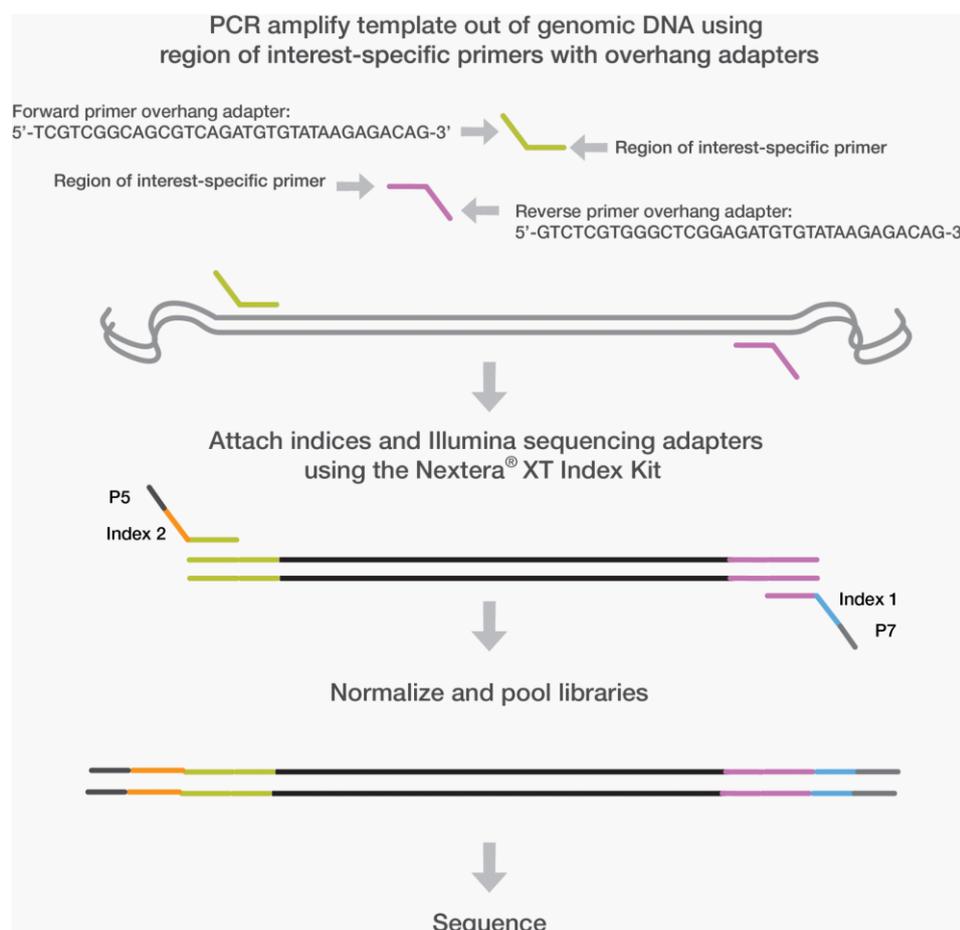
Figure 1.8. Illumina 16S rRNA microbiome profiling workflow.

1.4.4. Limitations of NGS platforms

The NGS based platforms are by no means a panacea for the assessment of gut microbial communities, and like all other scientific methods, they have their limitations. The available platforms currently lack the capacity to produce reliable full length reads of the 16S rRNA gene, however the PacBio system appears to be nearing this capability (Schloss et al 2016) and will likely achieve this aim in the near future. The shorter reads currently produced by NGS platforms can pose difficulties when assembling and mapping to reference sequences, particularly at repetitive regions. Short reads may also pose challenges in classifying bacteria, where a longer read may give more confidence when comparing against bacterial sequences from the various reference databases. As with any PCR-based molecular technology, amplification bias can also present some difficulties and may affect the estimated diversity indices. Sequencing errors, caused by repetitive sequences or homopolymers, are likely to be present in all of the NGS platforms, and error rates appear to escalate with increasing read length. Furthermore, non-specific amplification of host mitochondrial or plant chloroplast DNA may also occur. However, many of these difficulties can be minimized or circumvented by specific tools within the workflow of bioinformatic pipelines, which can detect and filter out problematic sequences.

Moreover, 16S rRNA gene copy numbers can vary from 1 to 15 copies per genome (Klappenbach et al 2001, Hugenholtz & Huber 2003). Therefore, NGS based approaches can underestimate the relative abundances of bacterial taxa with low 16S rRNA copy numbers and overestimate those containing multiple copies of the gene. Certain primer pairs may also exhibit amplification bias. Therefore, caution must be exercised when interpreting and especially when comparing surveys of microbial diversity using 16S rRNA based approaches. This has prompted some researchers to use alternative gene targets such as cpn60, which codes for bacterial chaperone complexes (Desai et al 2012). Finally, Illumina based platforms have experienced issues with the reverse read libraries of the 16S rRNA gene using the current V3 chemistry. However, computer programs such as FastQC (Andrews 2010) can help to overcome difficulties associated with these reads, by offering researchers a means of performing stringent quality control measures on sequencing files, post sequencing. If necessary, the program can be used to identify whether problematic reads require trimming in order to preserve sequence quality.

1.4.5. Investigations of the fish intestinal microbiome using NGS

Recent years have seen a large increase in the use of these technologies to explore the fish intestinal microbiome and to compare findings with those from mammals and terrestrial animals. As discussed above, bacterial diversity has been observed to progressively increase within the GI tract according to feeding habits, from carnivores to omnivores to herbivores in mammals (Ley et al 2008) and in a limited number of investigations of fish species (Ward et al 2009, Givens et al 2014, Larsen et al 2014, Li et al 2014). Larsen et al (2014) undertook a study of the intestinal microbiome of largemouth bass (*Micropterus salmoides*), channel catfish (*Ictalurus punctatus*), and bluegill (*Lepomis macrochirus*) using 454 sequencing, in order to compare the structure of these communities in carnivorous, omnivorous and herbivorous species, respectively. The authors reported the same trend as that reported in mammals, with the diversity of the intestinal microbiome being lowest in the carnivorous fish species. Givens et al (2014) used the same NGS platform and fed a single carnivorous Pinfish species, *Lagodon rhomboides*, three different diets, one consisting of krill, a second of seaweed and krill, and a third containing only seaweed. They found that the microbial diversity was greatest in the intestines of fish fed the seaweed-only diet, intermediate in the mixed diet, and lowest in the fish fed the krill-only diet. These results suggest that fish fed more herbivorous diets may support a greater degree of microbial diversity in their intestines, mirroring the patterns observed in mammalian species. The reasons for this are unclear, but could be related to the wider array of substrates within plant-based materials that can be preferentially metabolized by members of the intestinal microbiome. However, a different trend was reported by Bolnick et al (2014) in the three-spined stickleback (*Gasterosteus aculeatus*). In their experiments, diet diversity did not precipitate an increase in intestinal microbiome diversity. Thus, further research using NGS based approaches is required in order to explore the relationship between diet habit and the diversity of the intestinal microbiome in fish.

The limited number of NGS based studies on the intestinal microbiome of rainbow trout have revealed that dietary alterations induce varying effects on the structure of these communities. Wong et al (2013) used 454 pyrosequencing to reveal the diversity of the intestinal microbiome of these fish and to analyze the effect of both diet and stocking density on its composition. After feeding these fish two vastly different diets (grain based and fishmeal based) for 214 days, only slight differences were observed in the composition of the intestinal microbiome. A select group of bacterial taxa belonging to the phylum Firmicutes, particularly the genera *Lactobacillus*, *Clostridia* and *Streptococcus*, were observed to be discriminatory according to

diet, whilst the 'core' microbiome structure remained unaltered. Similarly, Desai et al (2012) used the same sequencing platform to examine the intestinal microbiome of trout fed plant and fishmeal based diets and reported an increase in bacteria belonging to the phylum Firmicutes in the fish fed plant based ingredients. Furthermore, Ingerslev et al (2014) used the Illumina HiSeq platform to analyze the effect of diets containing plant and marine based ingredients on first-feeding rainbow trout fry and reported a significantly higher abundance of the genera *Weissella*, *Streptococcus* and *Leuconostoc* in the intestinal microbiome of the fish fed the plant based diet. Zarkasi et al (2014) examined the impact of different commercial diets on the intestinal microbiome of adult farmed Atlantic salmon and reported very minor dietary effects on its composition. More recently, Mikyake et al (2015) reported a strong correlation between diet and the intestinal microbiome in different families of surgeonfishes (Acanthuridae) using 454 pyrosequencing. It is clear that further studies are required to investigate some of these initial findings, however the detection of shifts in minor components of the microbiome in response to dietary alterations strongly illustrates the utility of NGS approaches in addressing questions relating to the modulation of microbial populations in fish.

1.5. Hypotheses and objectives

The ultimate aim of this study is to investigate and characterize the distal intestinal microbiome of farmed rainbow trout. The thesis will test the hypotheses that that the intestinal microbiome is altered by varying dietary ingredients and differs according to farming environments. Furthermore, the potential existence of a 'core' distal intestinal microbiome will be investigated. Testing these hypotheses will contribute to improving our understanding of the complexity of the gut microbiota in trout and provide valuable baseline data on the natural composition of the intestinal microbiome in apparently healthy fish. The study will also enhance our knowledge of the relationship between diet and the intestinal microbiome and will elucidate some of the principal functional pathways expressed by these communities. Furthermore, this work serves to evaluate the use of next generation sequencing-based approaches in the phylogenetic and functional characterization of the intestinal microbiome of fish.

The objectives of this study are therefore:

1. To apply 16S rRNA based culture-independent methods to describe the composition and structure of the microbial flora in the distal intestine of farmed rainbow trout **(Chapter 2, 3, 4)**.
2. To establish detailed baselines for natural rainbow trout intestinal microbiomes **(Chapter 2, 3, 4)**.
3. To determine the structure of the microbial community in the intestine of fish fed different diet formulations **(Chapter 3)**.
4. To explore the extent to which microbial lineages are shared between individual fish in both aquarium and farm settings. In short, to investigate the hypothesized presence of a potential 'core' rainbow trout intestinal microbiome, that may be functionally beneficial to host health **(Chapter 4)**.
5. To investigate the principal functional pathways expressed by the intestinal microbiome of farmed rainbow trout **(Chapter 4)**.

1.6. References

Abid, A., Davies, S.J., Waines, P., Emery, M., Castex, M., Gioacchini, G., Carnevali, O., Bickerdike, R., Romero, J. and Merrifield, D.L. (2013) Dietary synbiotic application modulates Atlantic salmon (*Salmo salar*) intestinal microbial communities and intestinal immunity. *Fish & Shellfish Immunology* **35**, 1948-1956

Aguilera, E., Yany, G. and Romero, J. (2013) Cultivable intestinal microbiota of yellowtail juveniles (*Seriola lalandi*) in an aquaculture system. *Latin American Journal of Aquatic Research* **41**, 395-403

Aiso, K., Simidu, U. and Hasuo, K. (1968) Microflora in the digestive tract of inshore fish in Japan. *Journal of General Microbiology* **52**, 361-364

Andrews, S. (2010) FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Asakura, T., Sakata, K., Yoshida, S., Date, Y. and Kikuchi, J. (2014) Noninvasive analysis of metabolic changes following nutrient input into diverse fish species, as investigated by metabolic and microbial profiling approaches. *PeerJ* **2**: e550.10.7717/peerj.550

Asfie, M., Yoshijima, T. and Sugita, H. (2003) Characterization of the goldfish fecal microflora by the fluorescent in situ hybridization method. *Fisheries Science* **69**, 21-26

Baldo, L., Riera, J.L., Tooming-Klunderud, A., Alba, M.M. and Salzburger, W. (2015) Gut microbiota dynamics during dietary shift in eastern African cichlid fishes. *PLOS ONE* **10**, e0127462, **doi:** 10.1371/journal.pone.0127462

Behnke, R.J. (1992) *Native Trout of Western North America*. AFS Monographs 6. First Ed., American Fisheries Society, Bethesda, MA, USA

Bolnick, D.I., Snowberg, L.K., Hirsch, P.E., Lauber, C.L., Knight, R., Caporaso, J.G. and Svanback, R. (2014). Individuals' diet diversity influences gut microbial diversity in two freshwater fish (threespine stickleback and Eurasian perch). *Ecology Letters* **17**, 979-987

British Trout Association (BTA) (2016) History of Trout Farming. Available online at: http://britishtrout.co.uk/trot/farming-trout/history-of-trout-farming/

Buddington, R.K., Krogdahl, A. and Bakke-McKellep, A.M. (1997) The intestines of carnivorous fish: structure and functions and the relations with diet. *Acta Physiologica Scandinavica* **638**, 67-80

Buddington, R.K. and Kuz'mina, V. (2000) Digestive System. In: *The Laboratory Fish* (Ostrander, G.K. Ed.) pp.381-383, Academic Press, UK

Burr, G., Gatlin III, D.M. and Ricke, S. (2005) Microbial ecology of the gastrointestinal tract of fish and the potential application of prebiotics and probiotics in finfish aquaculture. *Journal of the World Aquaculture Society* **36**, 425-436

Cahill, M.M. (1990) Bacterial flora of fishes: A review. *Microbial Ecology* **19**, 21-41

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.Z., Costello, E.K., Fierer, N. et al (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**, 335-336

Carmody, R.N. and Turnbaugh, P.J. (2012) Gut microbes make for fattier fish. *Cell Host & Microbe* **12**, 259-261

Cerezuela, R., Fumanal, M., Tapia-Panigua, S.T., Meseguer, J., Morinigo, M.A. and Esteban, M.A. (2013a) Changes inintestinal morphology and microbiota caused by dietary administration of inulin and *Bacillus subtilis* in gilthead sea bream (*Spaurus auratus* L.) specimens. *Fish and Shellfish Immunology* **34**, 1063-1070

Cerezuela, R., Meseguer, J. and Esteban M.A. (2013b) Effects of dietary inulin, *Bacillus subtilis* and microalgae on intestinal gene expression in gilthead seabream (*Spaurus auratus* L.). *Fish and Shellfish Immunology* **34**, 843-848

Claridge III, J.E. (2004) Impact of 16S r RNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious disease. *Clinical Microbiology Reviews* **17**, 840-862

Clements, K.D. and Raubenheimer, D. (2005) Feeding and nutrition. In: *The Physiology of Fishes* (Evans, D.H. and Claiborne, J.B. Eds.) pp. 47-82, CRC Press, Boca Raton

Clements, K., Pasch, I., Moran, D. and Turner, S. (2007). Clostridia dominate the 16S rRNA gene libraries prepared from the hindgut of temperate marine herbivorous fishes. *Marine Biology* **150**, 1431-1440

Clements, K.D., Raubenheimer, D. and Choat, J.H. (2009) Nutritional ecology of marine herbivorous fishes: ten years on. *Functional Ecology* **23**, 79-92

Clements, K.D., Angert, E.R., Montgomery, W.L. and Choat, J.H. (2014) Intestinal microbiota in fishes: what's known and what's not. *Molecular Ecology* **23**, 1891-1898

Conway, P. (1989) Lactobacilli fact and fiction. In: *The Regulatory and Protective Role of the Normal Microflora* (Grubb, R., Midtvedt, T.M. and Norin, E. Eds.) pp. 263-281, Stockton Press, New York

Cowx, I.G. (2005) Cultured aquatic species information programme. *Oncorhynchus mykiss*. In: *FAO Fisheries and Aquaculture Department*, Rome, Italy. Available online at: http://www.fao.org/fishery/culturedspecies/Oncorhynchus_mykiss/en

Denev, S., Staykov, Y., Moutafchieva, R. and Beev, G. (2009) Microbial ecology of the gastrointestinal tract fish and the potential application of probiotics and prebiotics in finfish aquaculture. *International Aquatic Research* **1**, 1-29

Desai, A.R., Links, M.G., Collins, S.A., Mansfield, G.S., Drew, M.D., Van Kessel, A.G. and Hill, J.E. (2012) Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **350**, 134-142

Di Maiuta, N., Schwarzentruber, P., Schenker, M. and Schoelkopf, J. (2013). Microbial population dynamics in the faeces of wood-eating loricariid catfishes. *Letters in Applied Microbiology* **56**, 401-407

Dimitroglou, A. (2009) Effects of dietary mannanoligosaccharide (MOS) supplementation in relation to intestinal integrity, microbiota, health and production of cultured fish species. University of Plymouth Doctoral Thesis. Available online at: http://hdl.handle.net/10026.1/296.

Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C.L., Guroy, D. and Davies, S.J. (2011) Microbial manipulations to improve fish health and production: A Mediterranean perspective. *Fish and Shellfish Immunology* **30**, 1-16

Firouzbakhsh, F., Mehrabi, Z., Heydari, M., Kahlesi, M.J. and Tajick, M.A. (2012) Protective effects of a synbiotic against experimental *Saprolegnia parasitica* infection in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Research* **45**, 609-618

Fishelson, L., Montgomery, W.L. and Myrberg, A.A. (1985) A unique symbiosis in the gut of tropical herbivorous surgeonfish (Acanthuridae:Teleostei) from the Red Sea. *Science* **229**, 49-51

Frøystad, M.K., Lilleeng, E., Bakke-McKellep, A.M., Vekterud, K., Hemre, G.I. and Krogdahl, Å. (2008) Gene expression in distal intestine of Altlantic salmon (*Salmo salar* L.) fed genetically modified soybean meal. *Aquaculture Nutrition* **14**, 204-214

Fuglem, B., Jirillo, E., Bjerkås, I, Kiyono, H., Nochi, T., Yuki, Y., Raida, M., Fischer, U. and Koppang, E.O. (2010) Antigen sampling cells in the salmonid intestinal epithelium. *Developmental and Comparative Immunology* **34**, 768-774

Gatlin, D.M. III., Li, P., Wang, X., Burr, G.S., Castille, F. and Lawrence, A.L. (2006) Potential application of prebiotics in aquaculture. In: *Avances en Nutricion Acuicola VIII: VIII Simposium Internacional de Nutricion* (Suarez, E.C., Marie, D.R., Salazar, M.T., Lopez, M.G.N., Cavazos, D.A.V., Cruz, A.C.P. and Ortega, A.G. Eds.), pp. 371-376, Universidad Autonoma de Nuevo Leon, Monterrey, Nuevo Leon, Mexico

Ghanbari, M., Kneifel, W. and Domig, K. (2015) A new view of the fish gut microbiome: Advances from next-generation sequencing. *Aquaculture* **448**, 464-475

Gibson, G.R. and Roberfroid, M.B. (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of Nutrition* **125**, 1401-1405

Gibson, G.R., Probert, H.M., Loo, J.V., Rastall, R.A. and Robertfroid, M.B. (2004) Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews* **17**, 259-275

Gill, S.R., Pop, M., DeBoy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., Gordon, J.I., Relman, D.A., Fraser-Liggett, C.M. and Nelson, K.E. (2006) Metagenomic analysis of the human distal gut microbiome. *Science* **312**, 1355-1359

Givens, C.E., Ransom, B., Bano, N. and Hollibaugh, J.T. (2014) A fish tale: comparison of the gut microbiomes of 12 finfish and 3 shark species. *Journal of Marine Ecology Progression Series* **518**, 209-223

Goldin, B.R. (1986) In situ bacterial metabolism and colon mutagens. *Annual Reviews in Microbiology* **40**, 367-393

Goldin, B.R. and Gorbach, S.L. (1992) Probiotics for humans. In: *Probiotics, the Scientific Basis* (Fuller, R. Ed.) pp. 355-376, Chapman & Hall, London, UK

Goodrich, J.K., Di Rienzi, S.C., Poole, A.C., Koren, O., Walters, W.A., Caporaso, J.G., Knight, R. and Ley, R.E. (2014) Conducting a microbiome study. *Cell* **158**, 250-262

Gorbach, S.L. (1990) Lactic acid bacteria and human health. *Annals of Medicine* **22**, 7-41

Green, T.J., Smullen, R. and Barnes, A.C. (2013) Dietary soybean protein concentrate-induced intestinal disorder in marine farmed Atlantic salmon, *Salmo salar* is associated with alterations in gut microbiota. *Veterinary Microbiology* **166**, 286-292

Griffiths, S., Melvillea, K., Cooka, M., Vincenta, S., St. Maurice, P. and Lanteigne, C. (2001) Profiling of bacterial species associated with haddock larviculture by PCR amplification of 16S rDNA and denaturing gradient gel electrophoresis. *Journal of Aquatic Animal Health* **13**, 355-363

Guillaume, J. and Choubert, G. (1999) Digestive physiology and nutrient digestibility in fishes. In: *Nutrition and Feeding of Fish and Crustaceans*. (translated by Watson, J.) (Guillaume, G., Kaushik, S., Bergot, P. and Metailler, R. Eds.) pp. 27-58 Springer-Praxis Publishing, Chichester, UK

Haghighi, D.T., Fallahi, M. and Abdolahtabr, Y. (2010) The effects of different levels of Biomin® Imbo synbiotic on growth and survival of *Rutilis frisii kutum* fry. *Journal of Fisheries* **4**, 1-14

Hagi, T., Tanaka, D., Iwamura, Y. and Hoshino, T. (2004) Diversity and seasonal changes in lactic acid bacteria in the intestinal tract of cultured freshwater fish. *Aquaculture* **234**, 335-346

Hamer, H.M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F.J. and Brummer, R.J. (2008) Review article: the role of butyrate on colonic function. *Alimentary Pharmacology and Therapeutics* **27**, 104-119

Hansen, G.H. and Olafsen, J.A. (1999) Bacterial interactions in early life stages of marine cold water fish. *Microbial Ecology* **38**, 1-26

Hatha, A.A.M., Kuruvilla, S. and Cheriyan, S. (2000) Bacterial flora of the intestines of farm raised freshwater fishes *Catla catla*, *Labeo rohita* and *Ctenopharyngodon idella*. *Fishery Technology* **37**, 59-62

Hentschel, U., Horn, M., Friedrich, A.B., Wagner, M. and Moore, B.S. (2002) Molecular evidence for a uniform microbial community in sponges from different oceans. *Society* **68**, 4431-4440

Holben, W.E., Williams, P., Gilbert, M., Saarinen, M., Sarkilahti, L.K. and Apajalahti, J.H. (2002) Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. *Microbial Ecology* **44**, 175-185

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G. and Gordon, J.I. (2001) Molecular analysis of commensal host-microbial relationships in the intestine. *Science* **291**, 881-884

Hooper, L.V., Littman, D.R., and MacPherson, A.J. (2012) Interactions between the microbiota and the immune system. *Science* **336**, 1268-1273

Horn, M.H. (1997) Feeding and digestion. In: *The Physiology of Fishes* (Evans, D.H. Ed.), pp. 43-63. CRC Press, Boca Raton

Hugenholtz, P. and Huber, T. (2003) Chimeric 16S r DNA sequences of diverse origin are accumulating in the public databases. *International Journal of Systemic and Evolutionary Microbiology* **53**, 289-293

Ingerslev, H., von Gersdorff Jørgensen, L., Lenz Strube, M., Larsen, N., Dalsgaard, I., Boye, M. and Madsen, L. (2014). The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. *Aquaculture* **424**, 24-34

Inglis, G.D., Thomas, M.C., Thomas, D.K., Kalmokoff, M.L., Brooks, S.P.J. and Selinger, L.B. (2012) Molecular methods to measure intestinal bacteria: A review. *Journal of AOAC International* **95**, 5-23

Irianto, A. and Austin, B. (2002) Probiotics in aquaculture. *Journal of Fish Diseases* **25**, 633-642

Jonsson, E. and Conway, P. (1992) Probiotics for pigs. In: *Probiotics – The Scientific Basis* (Fuller, R. Ed.) pp. 259-316, Chapman and Hall, London

Jutfeld, F. (2006) The intestinal epithelium of salmonids: Transepithelial transport, barrier function and bacterial interactions. University of Göteborg, Sweden, Doctoral Thesis, pp. 8-20. Available online at:
http://www.bioenv.gu.se/digitalAssets/804/804535_Jutfeld_thesis_web.pdf

Kelly, D., Conway, S. and Aminov, R. (2005) Commensal gut bacteria: mechanisms of immune modulation. *Trends in Immunology* **26**, 326-333

Kim, D.H. and Austin, B. (2006) Innate immune responses in rainbow trout *Oncorhynchus mykiss* (Walbaum) induced by probiotics. *Fish and Shellfish Immunology* **21**, 513-524

Kim, D.H., Brunt, J. and Austin, B. (2007) Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). *Journal of Applied Microbiology* **102**, 1654-1664

Klappenbach, J.A., Saxman, P.R., Cole, J.R. and Schmidt, T.M. (2001) rrndb: the Ribosomal RNA operon copy number database. *Nucleic Acids Research* **29**, 181-184

Kostic, A.D., Howitt, M.R. and Garrett, W. (2013) Exploring host-microbiota interactions in animal models and humans. *Genes and Development* **27**, 701-718

Larsen, A.M., Mohammed, H.H. and Arias, C.R. (2014) Characterization of the gut microbiota of three commercially valuable warm water species. *Journal of Applied Microbiology* **116**, 1396-1404

Lau, W.W.Y., Jumars, P.A. and Armbrust, E.V. (2002) Genetic diversity of attached bacteria in the hindgut of the deposit-feeding shrimp *Neotrypaea* (formerly Callianassa) *californiensis* (Decapoda:Thalassinidae). *Microbial Ecology* **43**, 455-466

Lauzon, H.L., Dimitroglou, A., Merrifield, D.L., Ringø, E. and Davies, S.J. (2014) Probiotics and prebiotics: Concepts, definitions and history. In: *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. (1st edn.) (Merrifield, D. and Ringø, E. Eds.) pp. 170-184, WILEY Blackwell, Oxford, UK

Leaver, M., Villeneauve, L., Obach, A., Jensen, L., Bron, J. and Tocher, D. (2008) Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). *BMC Genomics* **9**: 299, **doi**: 10.1186/1471-2164-9-299

Lewis, J.D., Chen, E.Z., Baldassano, R.N., Otley, A.R., Griffiths, A.M., Lee, D., Bittinger, K., Bailey, A. et al (2015) Inflammation, antibiotics and diet as environmental stressors of the gut microbiome in pediatric Crohn's disease. *Cell Host and Microbe* **18**, 489-500

Ley, R.E., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R. and Bircher, S. (2008) Evolution of mammals and their gut microbes. *Science* **320**, 1647-1651

Li, X.M., Zhu, Y.J., Yan, Q.Y., Ringø, E. and Yang, D.G. (2014) Do the intestinal microbiotas differ between paddlefish (*Polyodon spathala*) and bighead carp (*Aristichthys nobilis*) reared in the same pond? *Journal of Applied Microbiology* **117**, 1245-1252

Li, T., Long, M., Gatesoupe, F.J., Zhang, Q., Li, A. and Gong, X. (2015) Comparative analysis of the intestinal bacterial communities in different species of carp by pyrosequencing. *Microbial Ecology* **69**, 25-36

Lindsay, G.J.H. and Gooday, G.W. (1985) Chitinolytic enzymes and the bacterial microflora in the digestive tract of cod, *Gadhus morhua*. *Journal of Fish Biology* **26**, 255-265

Liston, J. (1957) The occurrence and distribution of bacterial types on flatfish. *Journal of General Microbiology* **16**, 205-216

Liu, Z.Z., Lozupone, C., Hamady, M., Bushman, F. and Knight, R. (2007) Short pyrosequencing reads suffice for accurate microbial community analysis. *Nucleic Acids Research* **35**, e120

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H. and Derome, N. (2014) Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in Microbiology* **5**: 207, **doi:** 10.3389/fmicb.2014.00207

Llewellyn, M.S., McGinnity, P., Dionne, M., Letourneau, J., Thonier, F., Carvalho, G.R., Creer, S. and Derome, N. (2015) The biogeography of the Atlantic salmon (*Salmo salar*) gut microbiome. *The ISME Journal* **10**, 1280-1284

Lowrey, L., Woodhams, D.C., Tacchi, L. and Salinas, I. (2015) Topographical mapping of the rainbow trout microbiome reveals a diverse bacterial community in the skin with antifungal properties. *Applied and Environmental Microbiology* **81**, 6915-6925

Luo, C., Tsementzi, D., Kyrpides, N., Read, T. and Konstantinidis, K.T. (2012) Direct comparisons of Illumina vs. Roche 454 sequencing technologies on the same microbial community DNA sample. *PLOS ONE* **7**, e30087

MacFarlane-Stewart, M. (1932) The bacterial flora of the slime and intestinal contents of the Haddock (*Gadus aeglefinus*) *Journal of the Marine Biological Association of the United Kingdom* **18**, 35-50

MacFarlane, R., McLaughlin, J. and Bullock, G. (1986) Quantitative and qualitative studies of gut flora in striped bass from estuarine and coastal marine environments. *Journal of Wildlife Diseases* **22**, 344-348

Mansfield, G.S., Desai, A.R., Nilson, S.A., Van Kessel, A.G., Drew, M.D. and Hill, J.E. (2010) Characterization of rainbow trout (*Oncorhynchus mykiss*) intestinal microbiota and inflammatory marker gene expression in a recirculating aquaculture system. *Aquaculture* **307**, 95-104

Martin, S.A.M., Vilhelmsson, O., Medale, F., Watt, P., Kaushik, S. and Houlihan, D.F. (2003) Proteomic sensitivity to dietary manipulations in rainbow trout. *Biochimica et Biophysica Acta* **1651**, 17-29

Mazmanian, S.K. and Kasper, D.L. (2006) The love-hate relationship between bacterial polysaccharides and the host immune system. *Nature Reviews Immunology* **6**, 849-858

McFall-Ngai, M. (2014) Diving the essence of symbiosis: Insights from the squid-vibrio model. *PLOS Biology* **12**: e1001783, **doi:** 10.1371/journal.pbio.1001783

Mead, G.C. (1997) Bacteria in the gastrointestinal tract of birds. *Gastrointestinal Microbiology* **2**, 216-240

Merrifield, D.L., Dimitroglou, A., Bradley, G., Baker, R.T.M. and Davies, S.J. (2010a) Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) I. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria. *Aquaculture Nutrition* **16**, 504-510.

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R., Bøgwald, J., Castex, M. and Ringø, E. (2010b) The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* **302**, 1-18

Merrifield, D.L., Olsen, R.E., Myklebust, R. and Ringø, E. (2011) Dietary effects of soybean products on gut histology and microbiota of fish. In: *Soybean and Nutrition* (El-Shemy, H. Ed.) pp. 231-251, InTech, Croatia

Merrifield, D.L. and Carnevali, O. (2014) Probiotic modulation of the gut microbiota of fish. In: *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. (1st edn.) (Merrifield, D. and Ringø, E. Eds.) pp. 185-222, WILEY Blackwell, Oxford, UK

Merrifield, D.L., Balcazar, J.L., Daniels, C., Zhou, Z., Carnevali, O., Sun, Y.Z., Hoseinifar, S.H. and Ringø, E. (2014) Indigenous lactic acid bacteria in fish and crustaceans. In: *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. (1st edn.) (Merrifield, D. and Ringø, E. Eds.) pp. 128-168, WILEY Blackwell, Oxford, UK

Metzker, M.L. (2010) Sequencing technologies – the next generation. *Nature Reviews Genetics* **11**, 31-46

Miyake, S., Ngugi, D.K. and Stingl, U. (2015) Diet strongly influences the gut microbiota of surgeonfishes. *Molecular Ecology,* **24**, 656-672

Mizrahi-Man, O., Davenport, E.R. and Gilad, Y. (2013) Taxonomic classification of bacterial 16S rRNA genes using short sequencing reads: Evaluation of effective study designs. *PLOS ONE* **8**: e53608, **doi**: 10.1371/journal.pone.0053608

Montalban-Arques, A., De Schryver, P., Bossier, P., Gorkiewicz, G., Mulerio, V., Gatlin III, D.M. and Galindo-Villegas, J. (2015) Selective manipulation of the gut microbiota improves immune status in vertebrates. *Frontiers in Immunology* **6**: 512, **doi**: 10.3389/fimmu.2015.00512

Moore, W.E. and Holdeman, L.V. (1974) Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Applied Microbiology* **27**, 961-979

Morales, A.E., Cardenete, G., De La Higuera, M. and Sanz, A. (1994) Effects of dietary protein source on growth, feed conversion and energy utilization in rainbow trout *Oncorhynchus mykiss*. *Aquaculture* **124**, 117-126

Moran, D., Turner, S.J. and Clements, K.D. (2005) Ontogenetic development of the gastrointestinal microbiota in the marine herbivorous fish *Kyphosus sydneyanus*. *Microbial Ecology* **49**, 590-597

Mountfort, D.O., Campbell, J. and Clements, K.D. (2002). Hindgut fermentation in three species of marine herbivorous fish. *Applied and Environmental Microbiology* **68**, 1374-1380.

Muegge, B.D., Kuczynski, J., Knights, D., Clemente, J.C., Gonzalez, A., Fontana, L., Henrissat, B., Knight, R. and Gordon, J.I. (2011) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science* **332**, 970-974

Mundt, E., Collett, S.R., Berghaus, R., Pedroso, A.A., Lee, M.D. and Maurer, J.J. (2015) Can bacteriotherapy using commercially available probiotics, prebiotics and organic acids ameliorate the symptoms associated with runting-stunting syndrome in broiler chickens? *Avian Diseases* **59**, 201-206

Navarrete, P., Espejo, R.T. and Romero, J. (2009) Molecular analysis of microbiota along the digestive tract of juvenile Atlantic salmon (*Salmo salar* L.). *Microbial Ecology* **57**, 550-561

Navarrete, P., Magne, F., Araneda, C., Fuentes, P., Barros, L., Opazo, R., Espejo, R. and Romero, J. (2012) PCR-TTGE analysis of 16S rRNA from rainbow trout (*Oncorhynchus mykiss*) gut microbiota reveals host-specific communities of active bacteria. *PLOS ONE* **7**, e31335

Nayak, S.K. (2010) Role of gastrointestinal microbiota in fish. *Aquaculture Research* **41**, 1553-1573

Nekoubin, H., Gharedashi, E., Imanpour, M.R. and Asgharimoghadam, A. (2012) The influence of synbiotic (Biomin Imbo) on growth factors and survival rate of zebrafish (*Danio rerio*) larvae via supplementation with biomar. *Global Veterinaria* **8**, 503-506

Nelson, J.A., Wubah, D.A., Whitmert, M.E., Johnson, E.A. and Stewart, D.J. (1999) Wood-eating catfishes of the genus *Panaque*: gut microflora and cellulolytic enzyme activities. *Journal of Fish Biology* **54**, 1069-1082

Ni, J., Yan, Q., Yu, Y. and Zhang, T. (2014) Factors influencing the grass carp gut microbiome and its effect on metabolism. *FEMS Microbiology Ecology* **87**, 704-714

Nonogaki, H., Nelson, J. and Patterson, W. (2007) Dietary histories of herbivorous loricariid catfishes: Evidence from σ13C values of otoliths. *Environmental Biology of Fishes* **78**, 13-21

Oakley, B.B., Lillehoj, H.S., Kogut, M.H., Kim, W.K., Maurer, J.J., Pedroso, A., Lee, M.D., Collett, S.R., Johnson, T.J. and Cox, N.E. (2014) The chicken gastrointestinal microbiome. *FEMS Microbiology Letters* **360**, 100-112

O'Hara, A. and Shanahan, F. (2006) The gut flora as a forgotten organ. *EMBO Reports* **7**, 688-693

O'Mahony, C., Scully, P., O'Mahony, D., Murphy, S., O'Brien, F., Lyons, A., Sherlock, G., MacSharry, J., Kiely, B., Shanahan, F. and O'Mahony, L. (2008) Commensal-induced regulatory T-cells mediate protection against pathogen-stimulated NF-κB activation. *PLOS Pathogens* **4**, e1000112

Øyri, S.F., Muzes, G. and Sipos, F. (2015) Dysbiotic gut microbiome: A key element of Crohn's disease. *Comparative Immunology, Microbiology and Infectious Diseases* **43**, 36-49

Pettersen, E.F. (2003) Monoclonal antibodies to leucocytes and immunoglobulin from Atlantic salmon (*Salmo salar* L.) – Production, characterization and application. Department of Fisheries and Marine Biology. University of Bergen, Bergen, Norway.

Pond, M.J., Stone, D.M. and Alderman, D.J. (2006) Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **261**, 194-203

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S. and Medzhitov, R. (2004) Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* **118**, 229-241

Raida, M.K., Larsen, J.L., Nielsen, M.E. and Buchmann, K. (2003) Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of *Bacillus subtilis* and *B. licheniformis* (BioPlus2B). *Journal of Fish Diseases* **26**, 495-498

Rawls, J.F., Samuel, B.S. and Gordon, J.I. (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences USA* **101**, 4596-4601

Rawls, J.F., Mahowald, M.A., Ley, R.E. and Gordon, J.I. (2006) Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell* **127**, 423-433

Ray, A.K., Ghosh, K. and Ringø, E. (2012) Enzyme-producing bacteria isolated from fish gut: a review. *Aquaculture Nutrition* **18**, 465-492

Reed, G.B. and Spence, C.M. (1929) The intestinal and slime flora of the haddock: A preliminary report. *Contributions to Canadian Biology and Fisheries* **4**, 257-264

Ringø, E. (1993) The effect of chromic oxide (Cr_2O_3) on aerobic bacterial populations associated with the intestinal epithelial mucosa of arctic charr (*Salvelinus alpinus* L.). *Canadian Journal of Microbiology* **39**, 1169-1173

Ringø, E., Strom, E. and Tabachek, J. (1995) Intestinal microflora of salmonids: a review. *Aquaculture Research* **26**, 773-789

Ringø, E. and Gatesoupe, F.J. (1998) Lactic acid bacteria in fish: a review. *Aquaculture* **160**, 177-203

Ringø, E. and Birbeck, T.H. (1999) Intestinal microflora of fish larvae and fry. *Aquaculture Research* **30**, 73-93

Ringø, E., Lødemel, J.B., Myklebust, R., Kaino, T., Mayhew, T.M. and Olsen, R.E. (2001) Epithelium-associated bacteria in the gastrointestinal tract of Arctic charr (*Salvelinus alpinus* L.). An electron microscopical study. *Journal of Applied Microbiology* **90**, 294-300

Ringø, E., Olsen, R.E., Mayhew, T.M. and Myklebust, R. (2003) Electron microscopy of the intestinal microflora of fish. *Aquaculture* **227**, 395-415.

Ringø, E., Olsen, R.E., Gifstad, T Ø., Dalmo, R.A., Amlund, H., Hemre, G.I. and Bakke, A.M. (2010) Prebiotics in aquaculture: a review. *Aquaculture Nutrition* **16**, 117-136

Roeselers, G., Mittge, E., Stephens, W., Parichy, D., Cavanaugh, C., Guillemin, K. and Rawls, J.F. (2011) Evidence for a core gut microbiota in the zebrafish. *The ISME Journal* **5**, 1595-1608

Romero, J. and Navarrete, P. (2006) 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (*Oncorhynchus kisutch*). *Microbial Ecology* **51**, 422-430.

Romero, J., Ringø, E. and Merrifield, D. (2014) The gut microbiota of fish. In: *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. (1st edn.) (Merrifield, D. and Ringø, E. Eds.) pp. 75-100, WILEY Blackwell, Oxford, UK

Sakata, T., Nakaji, M. and Kakimoto, D. (1978) Microflora in the digestive tract of marine fish – I. General characterization of the isolates from yellowtail. *Memoirs of the Faculty of Fisheries of Kagoshima University* **27**, 65-71

Sakata, T., Uno, K. and Kakimoto, D. (1984) Dominant bacteria of the aerobic microflora in tilapia intestine. *Bulletin of the Japanese Society of Scientific Fisheries* **50**, 489-493

Sakata, T. (1990) Microflora in the digestive tract of fish and shellfish. In: *Microbiology in Poecilotherms* (Lesel, R. Ed.) pp. 171-176, Elsevier, Amsterdam

Schloss, P.D. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**, 7537-7541

Schloss, P.D., Jenior, M.L., Koumpouras, C.C., Westcott, S.L. and Highlander, S.K. (2016) Sequencing 16S rRNA gene fragments using the PacBio SMRT DNA sequencing system. *PeerJ* **4**:e1869

Sedgewick, S.D. (1990) *Trout Farming Handbook* (5th edn.). Fishing News Books, Blackwell Science, Oxford, UK

Seki, H. (1969) Marine microorganisms associated with the food of young salmon. *Applied Microbiology* **17**, 252-255

Semova, I., Carten, J.D., Stombaugh, J., Mackey, L.C., Knight, R., Farber, S.A. and Rawls, J.F. (2012) Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. *Cell Host & Microbe* **12**, 277-288

Sheehan, R.J., Shasteen, S.P., Suresh, A.V., Kapuscinski, A.R. and Seeb, J.E. (1999) Better growth in all-female diploid and triploid rainbow trout. *Transactions of the American Fisheries Society* **128**, 491-498

Siqueira Jr., J.F., Fouad, A.F. and Rocas, I.N. (2012) Pyrosequencing as a tool for better understanding of human microbiomes. *Journal of Oral Microbiology* **4**: 10.3402/jom.v4i0.10743

Sissons, J.W. (1989) Potential of probiotic organisms to prevent diarrhoea and promote digestion in farm animals: A review. *Journal of the Science of Food and Agriculture* **49**, 1-13

Skea, G.L., Mountfort, M.O. and Clements, K.D. (2005) A quantitative study of gut carbohydrases from the New Zealand herbivorous fishes *Kyphosus sydneyanus* (Kyphosidae), *Aplodactylus arctidens* (Aplodactylidae) and *Odax pullus* (Labridae). *Comparative Biochemistry and Physiology B* **140**, 259-269

Skea, G.L., Mountfort, D.O. and Clements, K.D. (2007) Contrasting digestive strategies in four New Zealand herbivorous fishes as reflected by carbohydrase activity profiles. *Comparative Biochemistry and Physiology A* **146**, 63-70

Smith, T.B., Wahl, D.H. and Mackie, R.I. (1996) Volatile fatty acids and anaerobic fermentation in temperate piscivorous and omnivorous freshwater fish. *Journal of Fish Biology* **48**, 829-841

Smriga, S., Sandin, S. and Azam, F. (2010) Abundance, diversity and activity of microbial assemblages associated with coral reef fish guts and feces. *FEMS Microbiology Ecology* **73**, 31-42

Stappenbeck, T., Hooper, L.V. and Gordon, J. (2002) Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proceedings of the National Academy of Sciences USA* **99**, 15451-15455

Stevens, C.E. and Hume, I.D. (1998) Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiological Reviews* **78**, 393-427

Sugita, H., Oshima, K., Tamura, M. and Deguchi, Y. (1983) Bacterial flora in the gastrointestine of freshwater fishes in the river. *Bulletin of the Japanese Society of Scientific Fisheries* **49**, 987-991

Sugita, H., Tokuyama, K. and Deguchi, Y. (1985) The intestinal microflora of carp *Cyprinus carpio*, grass carp *Ctenopharyngodon idella* and tilapia *Sarotherodon niloticus*. *Bulletin of the Japanese Society of Scientific Fisheries* **50**, 1325-1329

Sugita, H., Nakamura, T., Tanaka, K. and Deguchi, Y. (1994) Identification of Aeromonas species isolated from freshwater fish with the microplate hybridization method. *Applied and Environmental Microbiology* **60**, 3036-3038

Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'Connor, M.P., Rosen, G.L., Knight, R., Kilham, S.S. and Russell, J.A. (2012) Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Molecular Ecology* **21**, 3363-3378

Sun, H., Jami, E., Harpaz, S. and Mizrahi, I. (2013) Involvement of dietary salt in shaping bacterial communities in European sea bass (*Dicentrarchus labrax*). *Scientific Reports* **3**:1558, **doi**: 10.1038/srep01558

Sweetman, J.W., Torrecillas, S., Dimitroglou, A., Rider, S., Davies, S.J. and Izquierdo, M.S. (2010) Enhancing the natural defences and barrier protection of aquaculture species. *Aquaculture Research* **41**, 345-355

Tapia-Panigua, S.T., Reyes-Berrecil, M., Ascencio-Valle, F., Esteban, M.A., Clavijo, E., Balebona, M.C. and Morinigo, M.A. (2011) Modulation of the intestinal microbiota and immune system of farmed *Spaurus aurata* by the administration of the yeast *Debaryomyces hansenii* L2 in conjunction with inulin. *Journal of Aquaculture Research and Development* **S1**: 012, **doi**: 10.4172/2155-9546.S1-012

Trust, T.J., Bull, L.M., Currie, B.R. and Buckley, J.T. (1979) Obligate anaerobic bacteria in the gastrointestinal microflora of the grass carp (*Ctenopharyngodon idella*), goldfish (*Carassius auratus*), and rainbow trout (*Salmo gairdneri*). *Journal of the Fisheries Research Board of Canada* **36**, 1174-1179

Tsuchiya, C., Sakata, T. and Sugita, H. (2008) Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. *Letters in Applied Microbiology* **46**, 43-48

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R. and Gordon, J.I. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027-1031.

Turnbaugh, P.J. and Gordon, J.I. (2009) The core gut microbiome, energy balance and obesity. *The Journal of Physiology* **587**, 4153-4158.

Turnbaugh, P.J., Ridaura, V.K., Faith, J.J., Rey, F.E., Knight, R. and Gordon, J.I. (2009) The effect of diet on the human gut microbiome: A metagenomics analysis in humanized gnotobiotic mice. *Science Translational Medicine* **1**: 6ra14, **doi**: 10.1126/scitranslmed.3000322

Tveteras, R., Nystoyl, R. and Jory, D. (2015) Aquaculture production forecast. Global Aquaculture Alliance (GOAL) Conference, Vancouver, BC, Canada, October 2015

Uchii, K., Matsui, K., Yonekura, R., Tani, K., Kenzaka, T., Nasu, M. and Kawabata, Z. (2006) Genetic and physiological characterization of the intestinal bacterial microbiota of Bluegill (*Lepomis macrochirus*) with three different feeding habits. *Microbial Ecology* **51**, 277-284

Upadrasta, A., O'Sullivan, L., O'Sullivan, O., Sexton, N., Lawlor, P.G., Hill, C., Fitzgerald, G.F., Stanton, C. and Ross, R.P. (2013) The effect of dietary supplementation with spent cider yeast on the Swine distal gut microbiome. *PLOS ONE,* **8**, 75714

van Kessel, M., Dutilh, B., Neveling, K., Kwint, M., Veltman, J., Flik, G., Jetten, M., Klaren, P. and Op den Camp, H. (2011) Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (*Cyprinus carpio* L.) *AMB Express* **1**:41, **doi:** 10.1186/2191-0855-1-41

Verner-Jeffreys, D.W., Shields, R.J., Bricknell, I.R. and Birbeck, T.H. (2003) Changes in the gut-associated microflora during the development of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae in three British hatcheries. *Aquaculture* **219**, 21-42

Wang, H., He, M., Live, P., Hu, T. and Chen, T. (1994) Study on the intestinal microflora of carp in freshwater culture pond. *Acta Hydrobiologica* **18**, 354-359

Ward, N.L., Steven, B., Penn, K., Methe, B.A. and Detrich, W.H. (2009) Characterization of the intestinal microbiota of two Antarctic notothenioid fish species. *Extremophiles* **13**, 679-685

Woese, C.R. (1987) Bacterial evolution. *Microbiology and Molecular Biology Reviews* **51**, 221-271

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T., Wiens, G.D., Snekvik, K., Rawls, J.F. and Good, C. (2013) Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. *Applied and Environmental Microbiology* **79**, 4974-4984

Wu, S., Wang, G., Angert, E.R., Wang, W., Li, W. and Zou, H. (2012) Composition, diversity and origin of the bacterial community in grass carp intestine. *PLOS ONE* **7**, e30440

Wu, S.G., Tian, J.Y., Gatesoupe, F.J., Li, W.X., Zou, H., Yang, B.J. and Wang, G.T. (2013) Intestinal microbiota of gibel carp (*Carassius auratus gibelio*) and its origin as revealed by 454 pyrosequencing. *World Journal of Microbiology and Biotechnology* **29**, 1585-1595

Xing, M., Hou, Z., Yuan, J., Liu, Y., Qu, Y. and Liu, B. (2013) Taxonomic and functional metagenomics profiling of gastrointestinal tract microbiome of the farmed adult turbot (*Scopthalmus maximus*) *FEMS Microbiology Ecology* **86**, 432-443

Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F.O., Ludwig, W., Schleifer, K.H., Whitman, W.B., Euzeby, J., Amann, R. and Rosselló-Mora, R. (2014) Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nature Reviews Microbiology* **12**, 639-645

Yeoman, C.J. and White, B.A. (2014) Gastrointestinal tract microbiota and probiotics in production animals. *Annual Review of Animal Biosciences* **2**, 469-486

Yoshimizu, M. and Kimura, T. (1976) Study on the intestinal microflora of salmonids. *Fish Pathology* **10**, 243-259

Zarkasi, K., Abell, G., Taylor, R., Neuman, C., Hatje, E., Tamplin, M., Katouli, M. and Bowman, J.P. (2014) Pyrosequencing based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system. *Journal of Applied Microbiology* **117**, 18-27

Chapter 2

Exploring the microbial diversity of the distal intestinal lumen and mucosa of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) using next generation sequencing (NGS)

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2.1. Abstract

In this study, NGS was used to survey the 16S rRNA ribotypes of the distal intestinal lumen and mucosal epithelium of farmed rainbow trout. This approach yielded a library consisting of 2,979,715 quality filtered paired sequences, assigned to genus level of taxonomy using the Ribosomal Database Project (RDP). A high level of diversity was observed in both regions. A total of 90 bacterial genera were identified in the lumen of all fish sampled, compared with 159 in the mucosa. The allochthonous microflora was dominated by sequences belonging to the γ Proteobacteria (mean sequence abundance 54.3%), in particular the Enterobacteriaceae, with *Yersinia*, *Serratia*, *Hafnia* and *Obesumbacterium* the most abundant genera detected. Fewer γ Proteobacteria (mean sequence abundance 37%) were present in the mucosa, and autochthonous communities consisted of a more even split among the bacterial classes, with increases in sequences assigned to members of the β Proteobacteria (mean sequence abundance 18.4%) and Bacilli (mean sequence abundance 16.8%). The principal bacterial genera recorded in the mucosa were *Cetobacterium*, *Yersinia*, *Ralstonia*, *Hafnia* and *Carnobacterium*. The results of the present study demonstrate that the luminal and mucosal bacterial communities may be different in their respective structures, and that the mucosal microflora of rainbow trout may be more diverse than previous research has suggested. This research also demonstrated a degree of conservation of bacterial genera between individual fish sampled, and is to the author's knowledge the first time the MiSeq® NGS platform has been used to explore the rainbow trout intestinal microflora.

Keywords: aquaculture, bacteria, intestinal microflora, rainbow trout, 16S rRNA sequencing

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2.2. Introduction

The skin, gills, eggs and intestinal tracts of fish all harbour bacterial communities that are thought to impact upon their overall health through their interaction with these tissues. The GI tract in particular possesses a diverse microbial ecology that appears to vary among different fish species (Austin & Austin 1987, Cahill 1990, Ringø et al 1995, Merrifield et al 2009, Wu et al 2010, Di Maiuta et al 2013). In carnivorous fish, the distal intestinal region has been more extensively studied as it is considered to be an important site of nutrient absorption in the fish gut. This region is also believed to consist of a more stable microbial flora, owing to a lower level of exposure to bile salts, peristalsis, gastric acidity and digestive enzymes than the proximal region (Ringø et al 2003, Hartviksen et al 2014). The extent to which this microflora differs amongst apparently healthy individuals has been accurately characterized in humans (Guarner et al 2003, Turnbaugh et al 2006, Manichanh et al 2010, Durban et al 2011, Backhed et al 2005, 2012) but less so in fish.

The GI tract is thought to be a potential route of entry for many fish pathogens (Ringø & Birbeck 1999, Holben et al 2002, Salinas et al 2008). Research has suggested that the microbiota that occupy this mucosal layer of the brush border play a dual role, considered as a defensive barrier against pathogenic species in addition to aiding digestion via the production of a range of vitamins, short-chain fatty acids and enzymes (Ringø et al 1995, Nayak 2010). Many of the LAB are considered to be beneficial to the intestinal health of animals, and have been shown to exert a probiotic effect in the intestine of fish, including rainbow trout (Ringø & Gatesoupe 1998, Ringø 2008, Nayak 2010, Merrifield et al 2010a, b). There is further evidence that the GI microbiota possibly play a role in maintaining the integrity of the epithelial surface (Olsen et al 2002, Ringø et al 2003, 2007). Allochthonous, or transient, microbes may also have the ability to colonize the intestinal epithelium and positively contribute to host health. It is however possible that some of these transient microbes may be opportunistic pathogens who can only take advantage of a damaged or weak mucosal epithelium and thus negatively impact host homeostasis.

In rainbow trout, the dominant gut bacterial populations identified thus far belong to the β and γ subclass of Proteobacteria, especially the Enterobacteriaceae, Pseudomonadaceae and Vibrionaceae, but LAB have also been identified (Austin & Al-Zahrani 1988, Spanggaard et al 2000, Huber et al 2004, Heikkinen et al 2006, Pond et al 2006, Kim et al 2007). To date, the majority of attempts to characterize the microflora present in the fish intestine have focused on the use of bacterial culture-based methods combined with molecular-based techniques, especially DGGE and/or clone-library construction (Huber et al 2004, Pond et al 2006, Kim et al 2007, Merrifield et al 2009, Navarrete et al 2009, 2010, 2012). These studies have revealed that the fish gut does indeed harbour distinct bacterial communities. Techniques such as DGGE and clone-library construction have provided a snapshot of some of the dominant bacterial communities present in the GI tract of fish. However, these methods are limited in that they can only detect only a portion of the community members present in the intestinal environment, and their ability to semi quantitatively estimate these bacterial populations is restricted.

NGS platforms offer the possibility to explore the diversity of gut microbial communities on an unprecedented scale, and indeed have been principally used to study the microbial ecology of the human GI tract (Andersson et al 2008, Nam et al 2011, Morgan & Huttenhower 2012, Nakayama et al 2013). Numbers of bacterial 16S rRNA sequences derived from a sample can be assigned to individual genera/species thus providing a semi-quantitative estimation of the relative abundance of each microorganism present. Recently, Ingerslev et al (2014a, b) used this technology to profile the gut microbiota of juvenile rainbow trout and to accurately quantify shifts in intestinal microflora in response to *Y. ruckeri* infection. NGS platforms such as the Illumina MiSeq® can generate millions of paired-end microbial 16S rRNA gene sequences from a single sample, offering an extremely detailed picture of the structure of gut microbial communities and allowing researchers to test more complex hypotheses concerning the intestinal microflora than lower resolution molecular and culture-based methods.

A study was conducted to characterize the bacterial flora present in both gut content and mucous samples taken from the distal intestine of rainbow trout, using next-generation 16S rRNA sequencing performed on the Illumina MiSeq®. This platform has recently been used to characterize the gut microbial communities of cichlid fish (Franchini et al 2014), however the present study is the first to employ this method to study the rainbow trout intestinal microbiome. It is hypothesized that the intestinal contents and mucosal layer of the distal intestine harbour complex and distinct microbial communities, which may play different roles in contributing to the overall homeostasis of the GI tract and therefore fish health.

2.3. Materials and methods

- Sampling of intestinal content and mucous from 5 trout.

- DNA extraction, purification and quantification.

- Generate 16S rRNA library by PCR.

- Attach sequencing adapters by PCR.

- Normalize and pool 16S rRNA amplicon libraries.

- Cluster generation and sequencing.

- Phylogenetic assignment.
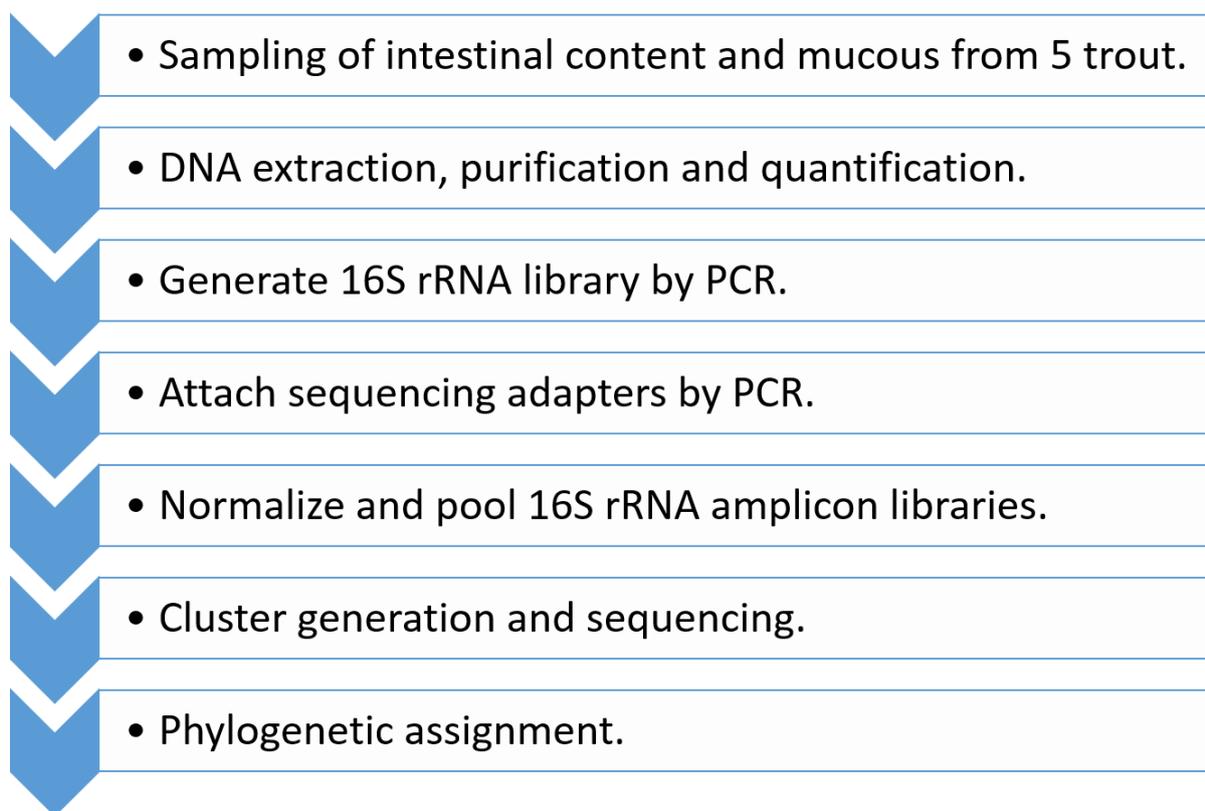
Figure 2.1. Flow chart showing the planned experimental approach of this study.

2.3.1. Sample collection and processing

The overall experimental approach of this study is depicted in Figure 2.1. Farmed diploid rainbow trout with an average weight of 191 ± 2.91g were obtained from a local trout farm (Perthshire, UK) and transferred to the Aquatic Research Facility (ARF) at the Institute of Aquaculture, Stirling University. The fish were kept in a 100 L tank maintained with a flow-through system and were fed twice a day on a commercial diet (Skretting UK). The light regime in the aquarium was 12 h light and 12 h dark. A total of 5 fish were sampled for both intestinal content and intestinal mucous from the hind-gut region of the digestive system. Fish were sacrificed with a high dose of the anaesthetic benzocaine (Sigma Aldrich®, Poole, UK) and were swabbed with 100% ethanol before dissection of the ventral surface (Figure 2.2). The tissues and visceral fat surrounding the digestive system were aseptically removed and the gut divided into fore-gut, mid-gut and hind-gut. The fore-gut refers to: the proximal portion of the digestive system including the esophagus and the stomach, the mid-gut: intermediate portion

of the digestive system including the pyloric caeca and small intestine, and the hind-gut: distal portion of the digestive system that corresponds to the large intestine (Hovda et al 2007). The distal gut contents, from a tissue section of approximately 2.5 cm in length, were removed by gently massaging the tissue with sterile forceps, and were placed directly into sterile 2 ml capped microtubes (Alpha laboratories®, Eastleigh, UK) containing 1 ml of lysis buffer (Qiagen®, Hilden, Germany). In order to separate luminal and mucosal communities, the tissue was then carefully dissected and the remaining contents removed by rinsing with a sterile 0.85% (w/v) saline solution. The intestinal mucous was removed by inverting a sterile scalpel and gently scraping the mucous from the gut wall. Mucous samples were similarly immersed in 1 ml of lysis buffer in sterile 2 ml capped microtubes and were immediately transferred to the laboratory on ice prior to DNA extraction.

Figure 2.2. Dissection of rainbow trout **a)** Swabbing ventral surface with 100% EtOH. **b)** Aseptic dissection of ventral surface to display viscera. **c)** Removal of 1cm of distal intestine anterior of vent to ensure sterility. **d)** Removal of distal intestinal contents. (D. Merrifield, personal communication 6th February 2013).

2.3.2. DNA extraction and purification

A total of ~150 mg of both intestinal contents and mucous samples from each individual fish suspended in 1 ml of buffer ASL (Qiagen) were processed for DNA extraction. Samples were firstly disrupted using a Mini bead-beater 16 (Biospec Ltd., Bartlesville, OK, USA) at maximum speed for four separate cycles of 35 s each. Samples were allowed to settle, and total genomic DNA was extracted and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), with the following modifications to the manufacturer's protocol – 150 mg starting material in 1 ml buffer ASL, and the suspension heated at 95°C for 10 min. Then, 0.5 Inhibitex tablet per sample was added to 700 µl supernatant and the final sample elution volume was adjusted to 50 µl. After extraction, DNA concentration of all samples was determined both spectrophotometrically (NanoDrop^{TM} 1000, Thermo Scientific, Glasgow, UK) and fluorometrically (Qubit$^{\circledR}$ Life Technologies, Paisley, UK) to ensure optimal measurement of DNA quantity and purity.

2.3.3. 16S rRNA PCR and Illumina sequencing

Illumina libraries were prepared following the method described by Caporaso et al (2012) using the NEXTflex 16S Amplicon-Seq kit (Bio Scientific, Austin, TX, USA). A total of 12.5 ng of template DNA was used for each individual sample and the V4 hypervariable region of the bacterial 16S rRNA gene (length 292bp) was amplified using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (GATC Biotechnology Inc., Konstanz). The PCR conditions were as follows; initial denaturation at 95°C for 5 min, 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s; followed by a final extension step at 72°C for 5 min. All samples were amplified in triplicate and all products purified using Agencourt Ampure XP beads (Beckman Coulter Ltd., Wycombe, UK). The products of the first PCR served as template for a second PCR with the same conditions as the first, however the number of cycles was reduced to eight, and Illumina sequencing adapters were added to the primers in the reaction mix. Following amplification, PCR products were visualized on a 1% agarose gel (Figure 2.3) and purified using Agencourt Ampure XP (Beckman Coulter) with a modified 1:1 volume of PCR product to Ampure XP beads. Purified amplicons were quantified with Qubit, pooled in equal concentration and the final quality of the pooled library was validated using a Bioanalyzer 2100 (Figure 2.4) (Agilent Technologies, Waldbronn, Germany). The final library was sequenced using the Illumina Mi Seq$^{\circledR}$ NGS system at GATC Biotechnology (Konstanz, Germany).
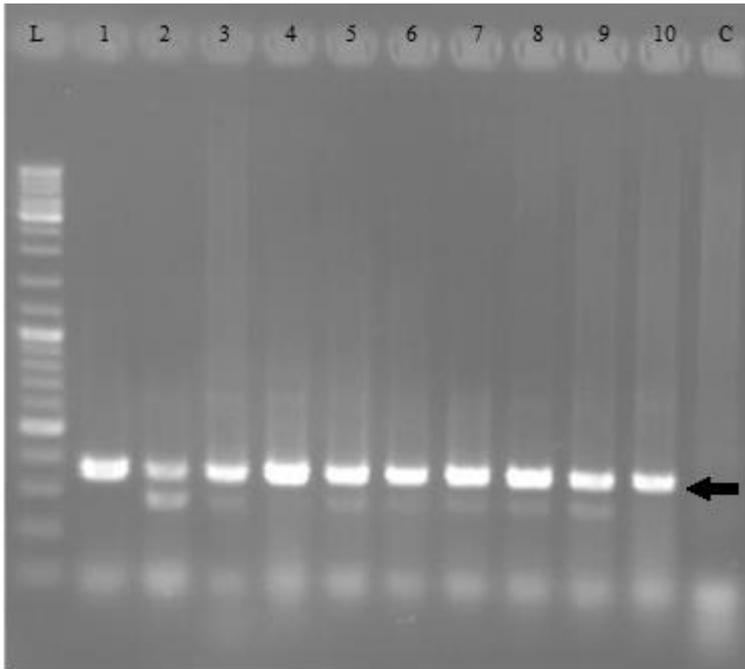
Figure 2.3. 1% Agarose gel electrophoresis after microbiome profiling PCR. PCR products visible as bands of approximately 350bp in size as indicated by arrow (after addition of overhang adapters). Lane L contains Generuler DNA ladder mix (Thermo Scientific®). Lanes 1-5 represent products generated from lumen samples (Fish 1-5). Lanes 6-10 are products generated from intestinal mucosa (Fish 1-5). Lane C represents negative control.
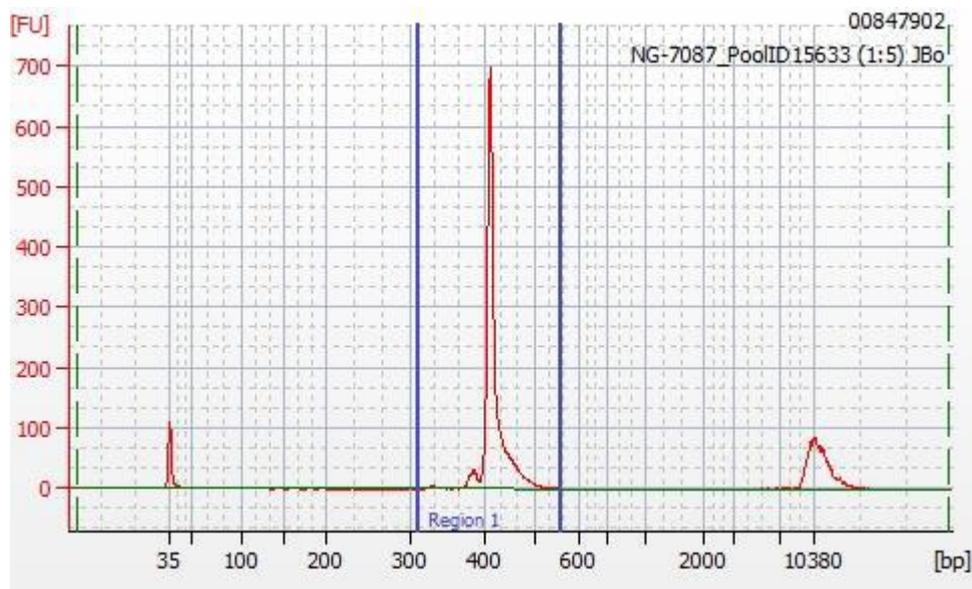
Figure 2.4. Agilent Bioanalyzer 2100 trace of final pooled amplicon library.

2.3.4. Bioinformatics

All 16S rRNA amplicons for each sample were compressed into individual fastq files. As paired-end amplicons are sequenced in both directions, the read pairs for each sample were merged based on overlapping bases using the fast length adjustment of short reads FLASh software tool (Magoc & Salzburg 2011) with a maximum mismatch density of 0.25. By merging read pairs in this way, the amplicon length is increased and the accuracy of downstream operational taxonomic unit (OTU) assignment is improved. The sequencing data was then compressed by performing sequence clustering based on 99% similarity accounting for PCR and sequencing errors (<1%). Sequence clustering was performed using the program cd-hit developed by Li & Godzik (2006). This step aids in minimizing differences between samples owing to variations in sequencing coverage. After clustering, singletons that had no other representation in the sequencing were discarded. UCHIME analysis was then performed on the clusters in order to remove chimeric clusters from the sequencing data from each sample.

Non-chimeric clusters were then subjected to basic local alignment search tool BLASTn analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using non-redundant 16S rRNA reference sequences with an E-value cutoff of 1e-06. Reference sequences were obtained from Ribosomal Database Project (RDP - version 10; Centre for Microbial Ecology, Michigan State University, East Lansing, MI, USA), and only full length (>1200bp) and unique rRNA sequences that had a taxonomic assignment were considered. In this way it was possible to assign OTU status to the clusters. Specific filters were then applied to the resulting clusters, including a >97% identity threshold, >95% alignment coverage and an e-value of <1e-06 in order to remove false positives. Finally, the number of reads within each cluster were consolidated in order to compute relative abundances. A full list of filters used during the bioinformatics analysis is provided in Table 2.1.

Table 2.1 – Description of filters used in bioinformatic analysis

Filter	Threshold
% Identity	≥97.00
E-value	≤1e-06
% Alignment coverage	≥95.00
Minimum query length	202
% Bitscore threshold for all hits	10
Maximum hits considered	50
% Abundance	>0.1

2.3.5. Analysis of bioinformatics data

Sequences that matched the same OTU were organized/binned into sequence clusters sharing very similar properties. The percentage of clusters hitting the same OTU was then calculated based on all of the OTU clusters recorded in the sample, thus representing the relative abundance of each OTU in each sample. Once the relative abundance of each OTU was calculated, these were grouped into phyla, classes and genera. The resulting relative abundances for each classifier in each individual fish were used to create charts depicting both the mean composition of bacterial phyla and classes in all fish analysed. Furthermore, the data was used to create a bubble plot, where the size of each bubble was correlated to the percentage relative abundance of clustered sequences attributed to each bacterial genus. In this way an individual microbial molecular fingerprint was created for each individual fish and intestinal region analysed.

2.3.6. Estimation of microbial diversity

Rarefaction curves, richness estimations and diversity indices were determined using the EstimateS (version 9.1.0 http://viceroy.eeb.uconn.edu/estimates/) and mothur (Schloss 2009) software tools, normalized to the sample with the lowest number of assigned read clusters at >97% sequence identity. The percentage coverage for each sample and diversity indices were determined by using the Inverse Simpson and Shannon commands within the EstimateS and mothur applications.

2.4. Results

2.4.1. Diversity and rarefaction analysis

Rarefaction and associated diversity analysis showed some variation in the level of microbial diversity between samples. The rarefaction analysis (Figure 2.5) however shows that the final number of OTU's per sample (ranging from 32 to 137) is not caused by uneven sequencing depth. This variation in detected OTU'S also appeared in diversity indices (Table 2.2). Most curves reached a saturation phase, and Good's coverage values of >98% were obtained for each individual sample, indicating a high level of sequence coverage (Table 2.2). From the calculated Shannon indices for each sample, two mucosal samples (F5M and F3M) showed the greatest microbial diversity, however the values obtained from the lumen samples are also indicative of a diverse community, in particular sample F2L. There were no statistically significant differences noted in diversity betwwen the two intestinal sites sampled, as $p > 0.05$ for two-sample t, one-way ANOVA and Kruskal Wallis tests respectively (Table 2.3).

Table 2.2 – Sequencing data and diversity indices of intestinal lumen (L) and mucous (M) samples

Sample	No. genera (OTU)	Total raw sequences	Total filtered sequences (Genus)	Shannon index	Inverse Simpson's Index	Good's coverage
F1 L	53	793740	209582	2.78	13.03	0.9992
F2 L	105	950341	95319	3.2	14.1	0.9960
F3 L	39	940454	419675	1.5	2.59	0.9992
F4 L	55	1337233	311723	2.99	10.92	0.9994
F5 L	54	930041	300027	2.98	12.64	0.9891
F1 M	32	1030291	284327	2.52	9.04	0.9999
F2 M	57	1252475	352580	3.16	16.12	0.9868
F3 M	109	622542	340232	3.45	17.5	0.9986
F4 M	47	2158233	516166	2.3	7.42	0.9998
F5 M	135	273826	150084	3.40	9.49	0.9921

Table 2.3 – Statistical analyses of alpha diversity metrics

Statistics	Shannon index	Inverse Simpson Index
Mean Lumen	2.69	10.66
Mean Mucosa	2.96	11.91
Two sample t-test P value	0.496	0.679
One-way ANOVA P value	0.494	0.677
Kruskal Wallis P value	0.465	0.917
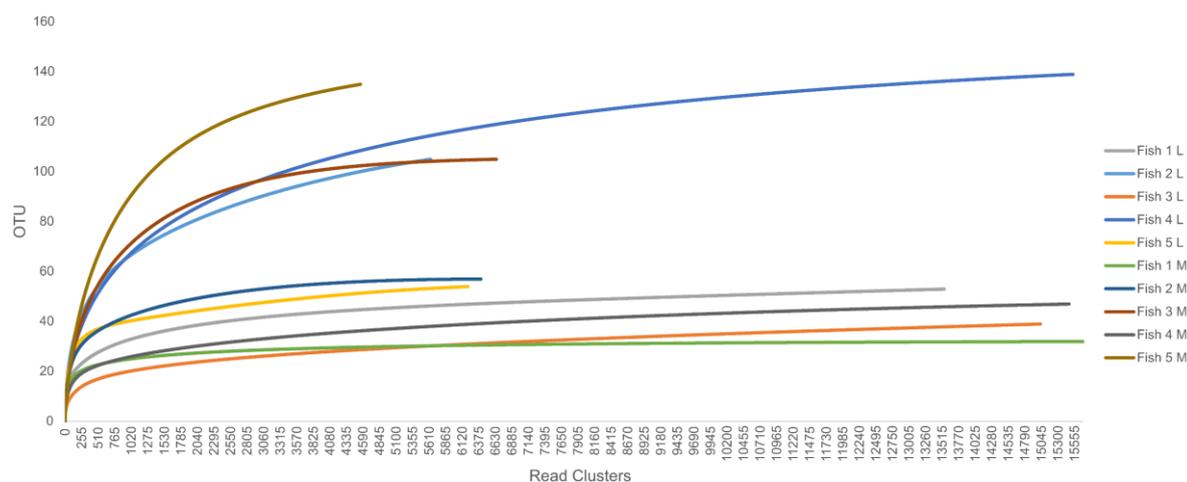
Figure 2.5. Rarefaction curves for each individual fish sampled, and at both the Lumen (L) and Mucosa (M). Curves represent the number of OTU's detected per sample as a function of the sequencing effort. Sequences are clustered using a pairwise similarity cut off of 97%.

2.4.2. Microbial diversity of rainbow trout intestinal lumen

Five 16S rRNA PCR libraries were generated, representing five samples of rainbow trout distal gut contents. After quality filtering, a total of 1,336,326 read pairs remained, each measuring 301bp, and were assigned to bacterial OTU's at a minimum sequence homology of 97% (an average of 267,265 sequences binned to 11,192 read clusters per sample). These read pairs clustered into five principal bacterial phyla. The majority of sequences belonged to members of the Proteobacteria (mean sequence abundance 58%), however members of the Fusobacteria (13%) and the Firmicutes (12%) were also well represented in the intestinal lumen. Members of the Bacteroidetes (0.3%) and Actinobacteria (0.2%) were present in much lower sequence abundances (Figure 2.6). In two fish (2 and 5), a high level of bacterial diversity and community evenness was noted when compared with the remaining three fish sampled. This was reflected in the apparent overall dominance of the γ Proteobacteria class in these three fish (Figure 2.7). The most abundant genera recorded, in terms of mean sequence abundance, were *Yersinia* (13.5%), *Serratia* (10.8%), *Hafnia* (9.3%), *Obesumbacterium* (6.8%) and *Cetobacterium* (7.6%). *Cetobacterium* is the only one of these four genera which is not part of the Enterobacteriaceae, but was frequently recorded in this study.

All genera recorded belonged to ten different bacterial classes (Figure 2.8). These genera were present in varying sequence abundances, and not in every fish sampled (Figure 2.7). Members

of the Gram positive LAB were present, with a high abundance of *Carnobacterium* (mean sequence abundance 4.15%), and were present in four out of five fish. Other members of the LAB detected in the samples were: *Streptococcus* (2.89%), *Lactobacillus* (1.15%), *Vagococcus* (0.72%), *Enterococcus* (0.69%), *Weissella* (0.39%), *Lacticigenium* (0.35%) and *Lactococcus* (0.23%). Further sequences belonging to the phylum Firmicutes that were detected included *Blautia* and *Veillonella*, of the classes Clostridia and Negativicutes respectively, but these were recorded in low sequence abundances (0.17% and 0.02%). Other genera that were commonly present were *Photobacterium*, *Rahnella*, *Ralstonia*, *Escherichia*, *Pseudomonas* and *Plesiomonas*. In the lumen samples, a large diversity of different genera was observed, with many of these being detected in very low levels of sequence abundance. Members of the phyla Bacteroidetes (*Sphingobacteria*, *Flavobacteria*) and Actinobacteria (*Corynebacterium*, *Micrococcus*) were poorly represented in lumen samples, making up only 0.3% and 0.2% respectively.
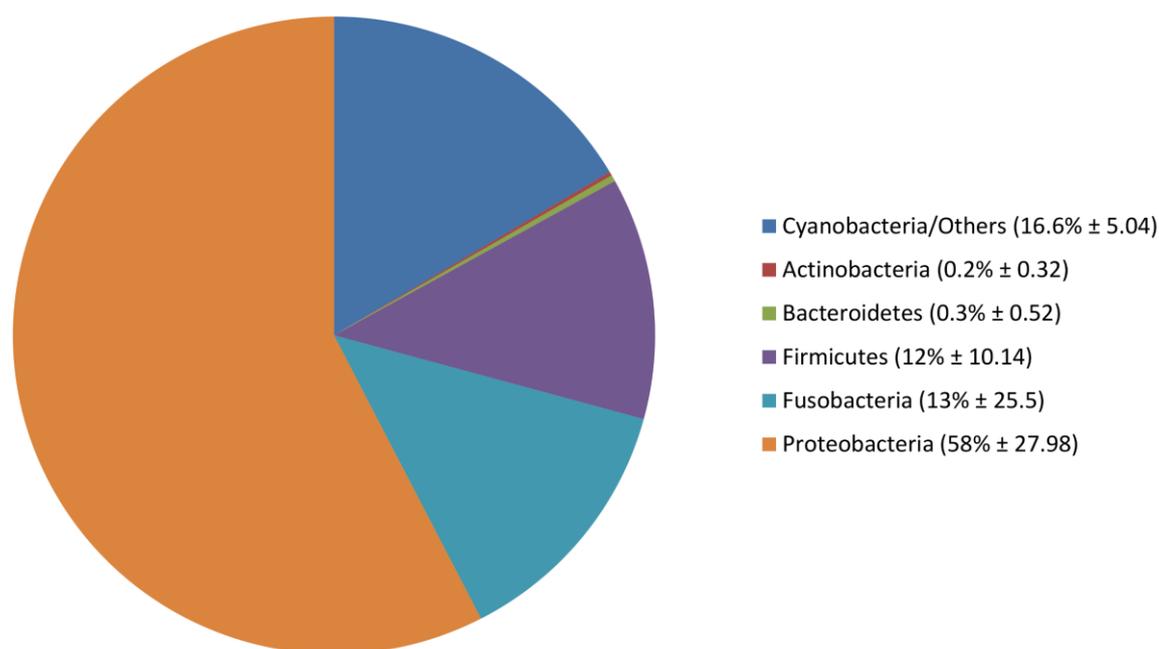
Figure 2.6. Composition of microbial phyla observed in rainbow trout intestinal lumen (% mean sequence abundance; n=5).
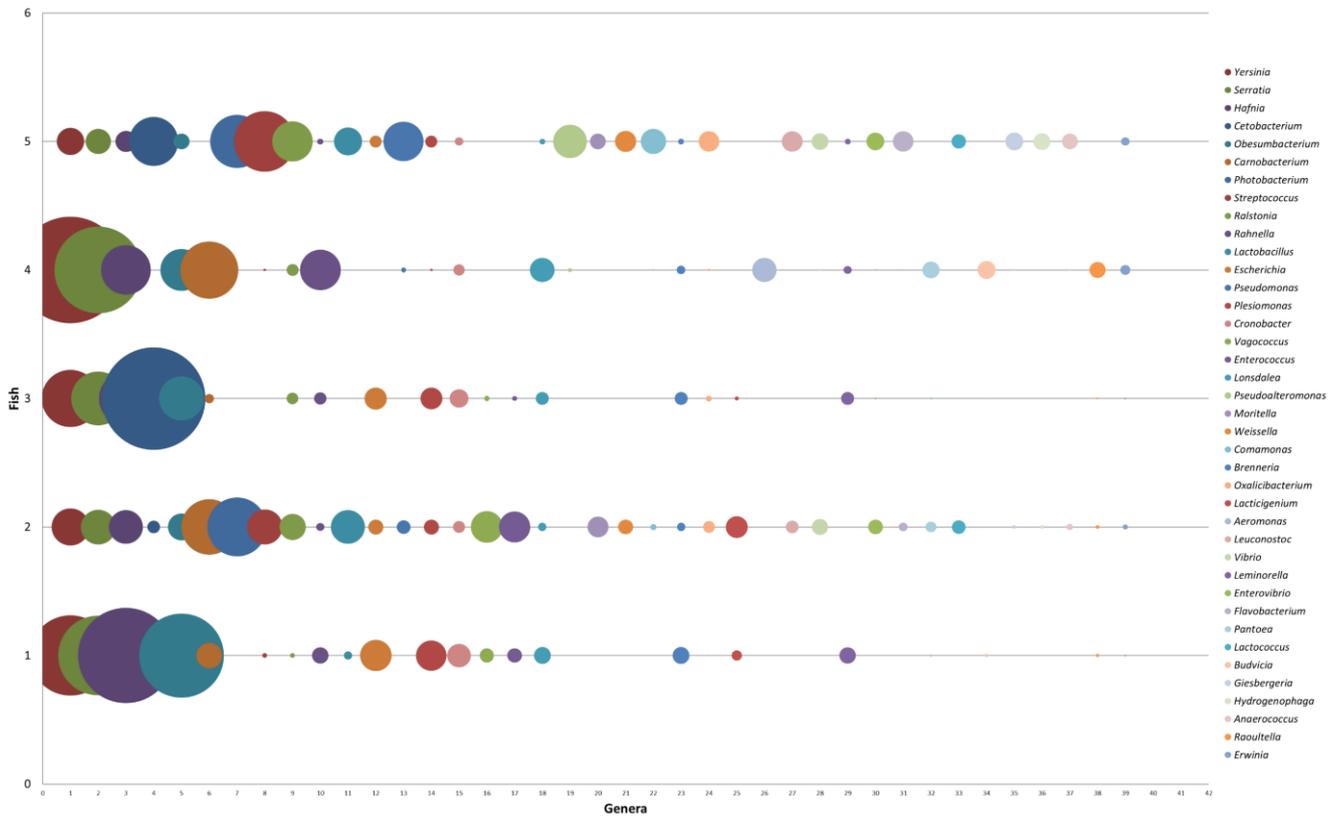
Figure 2.7. Microbial community composition of intestinal lumen for each individual fish sampled. As depicted in the key, bubble size is positively correlated with the relative percentage of sequence clusters attributed to each bacterial genus identified and thus the relative % abundance of sequences recorded in each sample. The y axis indicates individual fish The x axis represents bacterial genera. Sequence abundances <0.1% not shown.
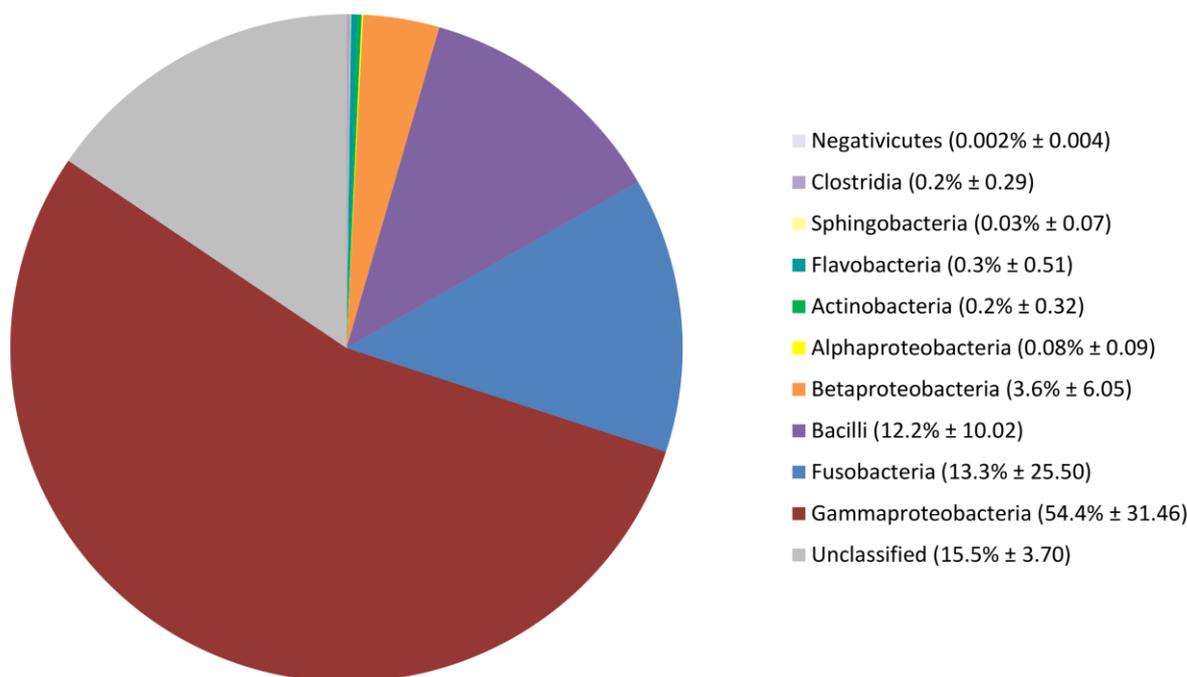
Figure 2.8. Composition of microbial classes observed in rainbow trout intestinal lumen (% mean sequence abundance; n=5).

2.4.3. Microbial diversity of rainbow trout intestinal mucosa

A further five 16S rRNA libraries were generated from template genomic DNA originating from rainbow trout intestinal mucous. A higher number of reads were obtained from these samples, providing a total of 1,643,389 after quality filtering (an average of 328,677 sequences binned to 9,754 read clusters per individual). The Proteobacteria were the most abundant (mean sequence abundance 62%) (Figure 2.9), however, within this phylum was an increase in the abundance of bacteria belonging to the classes α Proteobacteria (7%) and β Proteobacteria (18.4%), whereas a decrease in γ Proteobacteria was observed (37%) when compared with the lumen samples (Figure 2.10). There was also an increase in sequence clusters attributed to the Firmicutes in the mucosal samples. The Bacilli (16.8%) were the major class present, in addition to the Clostridia and Negativicutes, which were poorly represented at 0.6% and 0.1%, respectively. Furthermore, there was an increase in the number of sequences attributed to the phyla Actinobacteria (3%) and Bacteroidetes (2%). The abundance of Fusobacteria (10.8%) sequences was however lower in the mucous libraries. The most abundant genera recorded were *Cetobacterium* (10.7%), *Yersinia* (7.3%), *Ralstonia* (9.9%), *Hafnia* (6.6%) and *Carnobacterium* (6.2%).

A very diverse collection of microbial genera was observed in the mucosal layer of each fish, and a higher number of bacterial genera were detected when compared with the lumen (Figure 2.11). The Firmicutes were present in greater abundances than in the intestinal lumen. *Carnobacterium* was the most abundant genus of the LAB (6.2%), followed by, in order of decreasing mean abundance, *Staphylococcus* (2.7%), *Streptococcus* (2.3%), *Vagococcus* (1.74%), *Enterococcus* (1.72%) and *Lacticigenium* (0.9%). Other Gram positive bacteria that were detected included the genera *Arthrobacter* (0.8%), *Corynebacterium* (0.7%) and *Clostridium* (0.63%). *Lactococcus*, *Weissella* and *Lactobacillus* were present in low abundances (0.2%, 0.1% and 0.02% respectively). There was a notable presence of members of the order *Burkholderia* in the mucosal layer of all fish. In particular, a large increase was recorded in the numbers of reads attributed to the genus *Ralstonia*, when compared with intestinal lumen samples (9.92% mean sequence cluster abundance in mucous; 1.52% in lumen). Further members of this family included the genera *Oxalicibacterium* (2%) and *Massilia* (1.23%). The family *Bradyrhizobiaceae* was also represented (2.45%). Other notable microbial genera present in these samples were *Serratia* (6.5%), *Obesumbacterium* (4.8%) and *Pseudomonas* (2.5%). A number of genera belonging to the phylum Bacteroidetes were also present, albeit in lower numbers of sequences. *Flavobacteria* and *Sphingobacteria* represented 1.5 and 0.03% of mean sequence clusters respectively for all samples of intestinal mucous. The metabolism of many of the bacterial genera detected, in addition to their preferred niches and potential functions as documented in the literature, are outlined in Table 2.4 (S1).
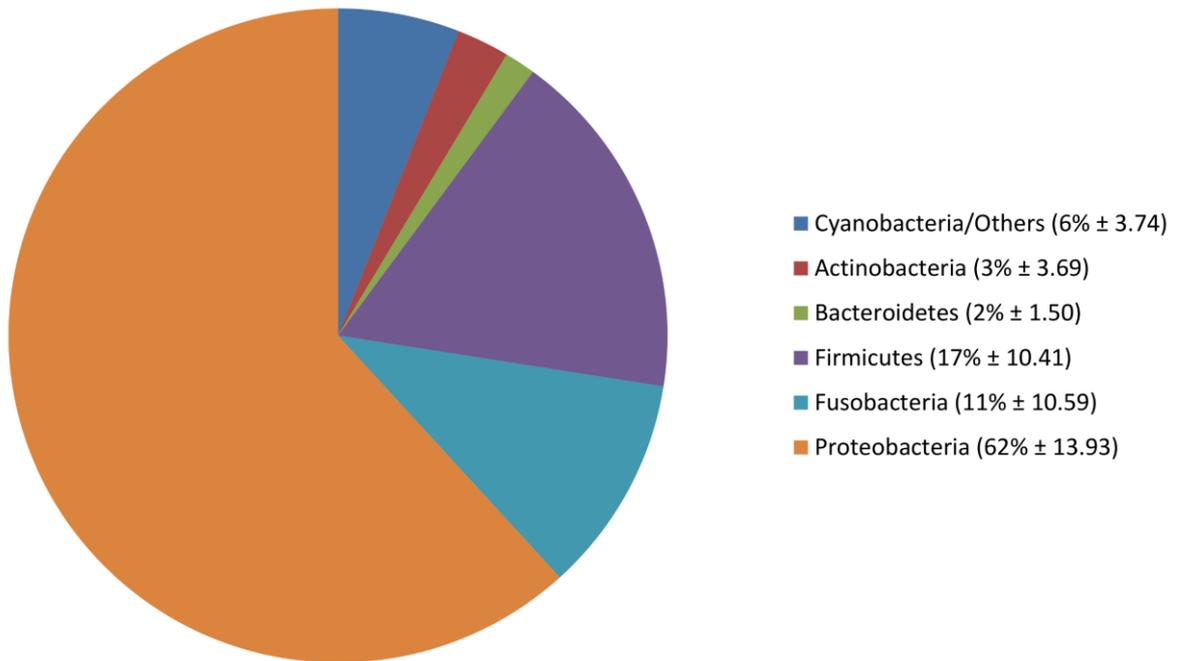
Figure 2.9. Composition of microbial phyla observed in rainbow trout intestinal mucosa (% mean sequence abundance; n=5).

Cyanobacteria/Others (6% ± 3.74)
Actinobacteria (3% ± 3.69)
Bacteroidetes (2% ± 1.50)
Firmicutes (17% ± 10.41)
Fusobacteria (11% ± 10.59)
Proteobacteria (62% ± 13.93)
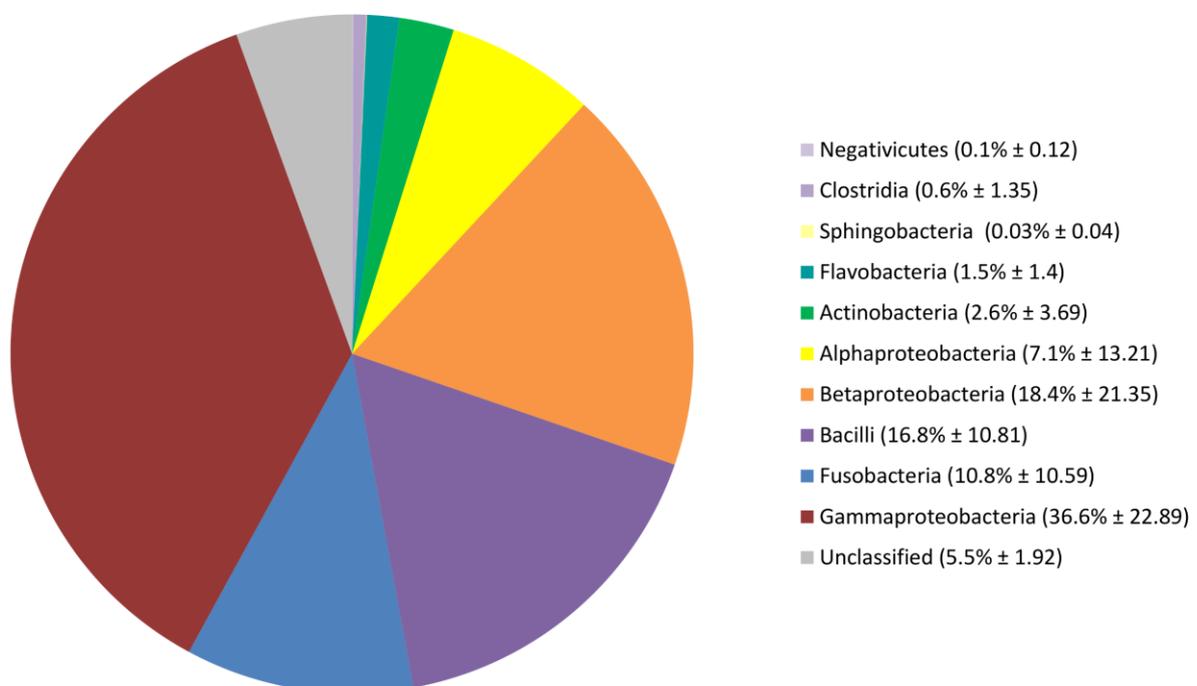
Figure 2.10. Composition of microbial classes observed in rainbow trout intestinal mucous (% mean sequence abundance; n=5).

Negativicutes (0.1% ± 0.12)
Clostridia (0.6% ± 1.35)
Sphingobacteria (0.03% ± 0.04)
Flavobacteria (1.5% ± 1.4)
Actinobacteria (2.6% ± 3.69)
Alphaproteobacteria (7.1% ± 13.21)
Betaproteobacteria (18.4% ± 21.35)
Bacilli (16.8% ± 10.81)
Fusobacteria (10.8% ± 10.59)
Gammaproteobacteria (36.6% ± 22.89)
Unclassified (5.5% ± 1.92)
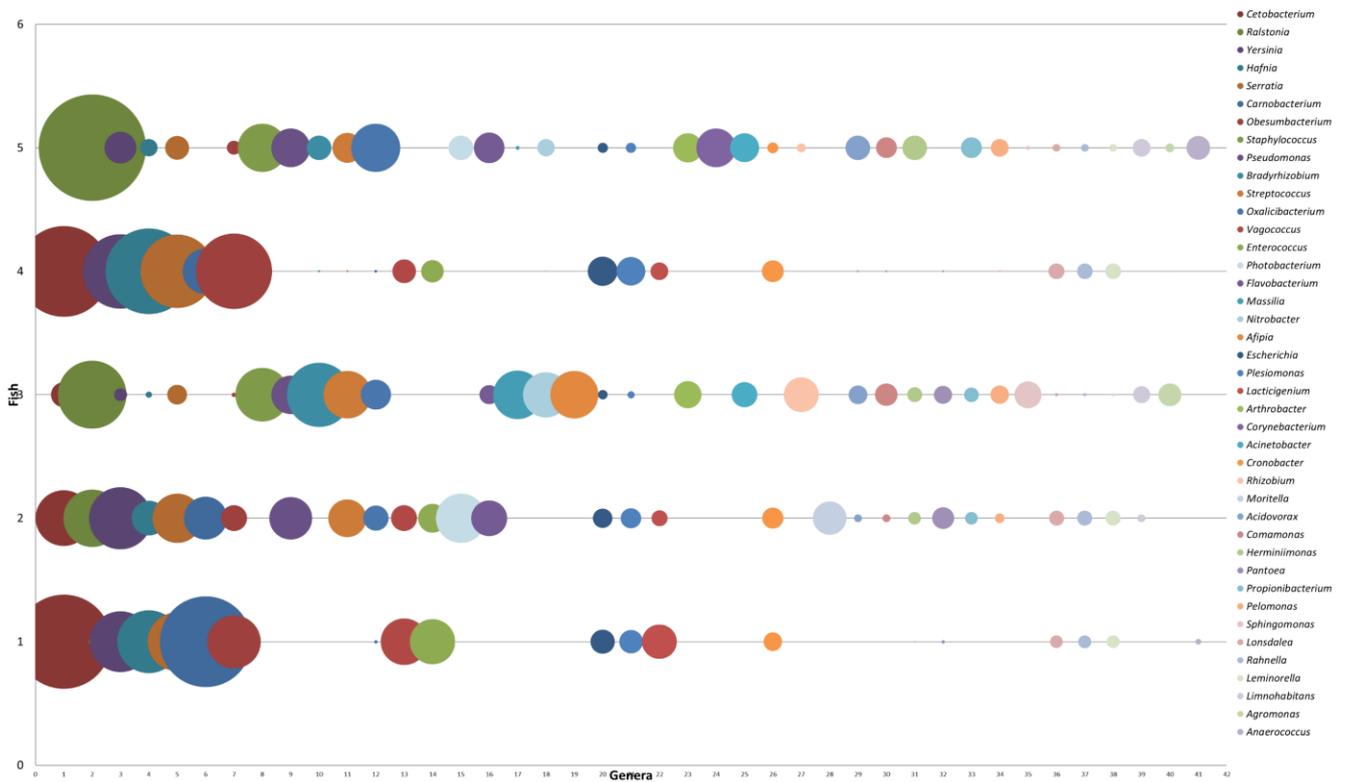
Figure 2.11. Microbial community composition of intestinal mucous of each individual fish sampled. As depicted in the key, bubble size is positively correlated with the relative percentage of sequence clusters attributed to each bacterial genus identified and thus the relative % abundance of sequences recorded in each sample. The y axis indicates individual fish. The x axis represents bacterial genera. Sequence abundances <0.1% not shown.

2.5. Discussion

The present study demonstrated, to the author's knowledge, the first successful application of NGS using the MiSeq® to investigate and characterize the microbial communities present in both the lumen and mucosal layer of the rainbow trout distal intestine. The data presented here suggest that these two regions of the intestine may possess structurally different microfloras, and that both regions harbour a very diverse microbiome. Differences in the diversity of bacterial genera present in the intestinal lumen and mucosal layer of the fish intestine have been recorded previously, with some studies noting a decreased microbial diversity in the mucosa, possibly owing to a limited ability to colonize the intestinal epithelium (Kim et al 2007, Merrifield et al 2009, Wu et al 2010). Indeed, similar trends have also been recorded in humans and terrestrial animals (Durban et al 2011). However, more recent research by Carda-Dieguez et al (2014) and Li et al (2014) reported a greater microbial diversity in the intestinal mucous of European sea bass (*Dicentrarchus labrax*) and grass carp (*Ctenopharyngodon idellus*) respectively, when compared with the lumenal communities of these fish, suggesting that the mucosal epithelium may in fact be host to a complex microbiome.

The distal intestine is considered to be the primary site of intestinal absorption of macromolecules in salmonids (Ringø et al 2003, Desai et al 2012). The microbiome of this region was therefore selected for analysis in this study in order to gain a comprehensive overview of the diversity of the microbial genera that may be present as both transient (lumen) and attached (mucosa) communities. At present, most research efforts have focused on the effects of dietary alterations on the fish gut microflora, assessing pooled samples from groups of fish fed differing dietary regimes. This practice can paint a very biased and inaccurate picture of the actual microbial community structure within individual fish, which may vary considerably. By addressing this level of variability, it may be possible to determine the extent to which microbial lineages are shared, and thus identify a potential 'core microbiota' (Roeselers et al 2011, Wong et al 2013). The function of this potential core requires further investigation, particularly in terms of the microbial influence on fish health and nutritional competency. The presented work was limited in this respect by the dual factors of a small sample size and the fact that it was aquarium based, however the bubble plot data offer a detailed molecular 'fingerprint' of the individual variations in the structure of bacterial communities between the different intestinal regions in each individual fish. The microbial community patterns detected point to a possible common molecular fingerprint, or core, within the small population of fish sampled. These individual molecular fingerprints were dominated

by sequences belonging to a select group of microbes, principally members of the Enterobacteriaceae, Leuconostocaceae and Fusobacteriaceae, whilst the remainder of sequences were attributed to apparently sporadic or rare organisms. This colonization pattern, with the dominance of a few abundant bacterial genera, and the majority of other genera being detected at low frequencies has been noted in previous studies of the fish intestinal microflora (Roeselers et al 2011, van Kessel et al 2011, Di Maiuta et al 2013, Franchini et al 2014, Xia et al 2014, Zarkasi et al 2014).

Both of the regions sampled in this study were highly diverse. Whilst we support that this degree of bacterial diversity should be carefully considered, a high level of diversity may be beneficial as it is generally thought to be desirable for ecosystem stability. Backhed et al (2005) referred to this concept as the 'Insurance hypothesis', which considers that a high level of microbial diversity in a given environment can confer resilience through a wider range of potential responses to stressful events within that environment. A balanced gut microbial ecosystem is therefore considered to be essential for host health (Upadrasta et al 2013) and there is some evidence linking low microbial diversity to human diseases such as obesity (Turnbaugh et al 2006, Turnbaugh & Gordon 2009, Langille et al 2013, Parks et al 2013). In the human GI tract, populations are quite stable within individuals, implying that mechanisms exist to suppress blooms of subpopulations and/or to promote the abundance of desirable bacteria. The collective genome of these 'desirable' bacteria must be complex enough to promote resilience in the intestinal ecosystem. It is possible that similar mechanisms are active in the intestinal tract of fish. Bolnick et al (2014) recorded conflicting results when examining the relationship between gut microbial diversity and host condition (relative mass) in two fish species (perch and stickleback) using NGS, reporting both positive and negative correlations between the two parameters. In the present study, different degrees of diversity were recorded between individual fish and indeed between individual regions of the rainbow trout intestine, however all of the fish sampled were apparently healthy and of a very similar weight. Further research is therefore required in order to more thoroughly examine the relationship between intestinal microbial diversity and fish health status.

The composition of bacterial classes recorded in this study is in broad agreement with previous explorations of the rainbow trout intestinal microflora, with the majority of bacterial genera belonging to the classes γ Proteobacteria, β Proteobacteria and Firmicutes (Kim et al 2007, Ingerslev et al 2014a). In the data presented, the distribution pattern of each of the 10 classes recorded was markedly different between and within the two intestinal regions analysed, and

in particular the mean relative abundance of bacterial sequences belonging to the γ Proteobacteria, which differed between the lumen and mucosa, representing 54% and 37%, respectively. This may suggest that a proportion of the microbes belonging to this class have difficulty colonizing the mucosal epithelium and thus may not be interacting directly with it. It could also be the case that the detection of higher numbers of γ Proteobacteria sequences reflects a potential role of these microbes as competitive dominants in the intestinal lumen. Furthermore, the decrease in members of this class in the mucosa was accompanied by an increase in sequences belonging to eight of the nine remaining classes, in particular the β Proteobacteria and Bacilli.

These results could indicate that certain members of these classes do not readily reproduce in the intestinal lumen, or are competitively excluded, but can colonize the epithelium. This is further supported by the increased sequence abundance of members of the α Proteobacteria, Actinobacteria and Bacteroidetes in the intestinal mucous samples of all fish examined, and some recent evidence has suggested that *Corynebacterium*, a member of the Actinobacteria, is commonly present in the mucosal layer of the healthy rainbow trout intestine (Hartviksen et al 2014, Ingerslev et al 2014a, b). Sequences attributed to Fusobacteria were slightly higher in the lumen samples, however this phylum was dominated by the genus *Cetobacterium*, which was more abundant in the mucous, albeit only slightly. The genus *Carnobacterium* was the most numerous of the LAB genera detected, and has previously been reported to be part of the healthy rainbow trout intestinal microflora. Some members of the Carnobacteriaceae have also shown potential as probiotic organisms (Austin 2006, Balcazar et al 2007, 2008, Kim & Austin 2008, Ringø 2008, Merrifield et al 2010a, b, c). *Ralstonia* was prominent in the mucosal samples, and this organism has also recently been reported as abundant in European sea bass distal intestinal mucosa (Carda-Dieguez et al 2014), in both gut content and mucous from yellow catfish (Wu et al 2010) and also in the intestinal contents of rainbow trout (Kim et al 2007). Members of the genus *Yersinia* were dominant in both regions, however these sequences were mostly assigned to the subtypes *Y. aldovae*, *Y. enterocolitica*, *Y. frederiksenii*, *Y. intermedia* and *Y. kristensii*, which have been reported as commensal intestinal organisms of many animals, including fish (Chen et al 2010). Few sequences were assigned to *Y. ruckeri*, the pathogen responsible for enteric red mouth (ERM) disease in salmonids. Nevertheless, the presence of *Y. ruckeri* could suggest that some of these fish could be latent carriers of this organism, and indeed the GI tract has been suggested as a possible portal of entry for this pathogen in fish (Nayak 2010).

Sequences assigned to obligate anaerobes in both the lumen and mucosal samples were detected at very low levels of abundance, with the exception of *Cetobacterium*. Nonetheless, a greater number of sequences attributed to obligate anaerobes in the mucosal layer may suggest that these microbes are functionally adapted to life in the intestinal ecosystem, increasing the possibility that these organisms are true mutualists. *Porphyromonas* was detected in the mucous samples at very low levels, but not in lumenal samples. Carda-Dieguez et al (2014) identified the Porphyromonadaceae as a key bacterial class in the intestinal mucous of cultured sea bass and indeed this class has frequently been reported in the GI tract of humans. Other obligate anaerobes such as *Propionibacterium* and *Sphingomonas*, whilst rare, were more abundant in the mucous samples analysed in the present study. The numbers of sequences attributed to these two genera were also similar to those observed in sea bass intestinal mucosa by Carda-Dieguez et al (2014) and in gilthead sea bream *Spaurus aurata* (Floris et al 2013). Both genera have been recorded in the GI tract of humans and terrestrial animals (Zarate et al 2004). The presence of *Propionibacterium* is notable as this bacterium has a fermentative metabolism and is also one of the chief microbes used in the industrial production of vitamin B12. This enhances the possibility that this microbe may play a probiotic role as a mutualist in the distal intestine of the rainbow trout. Furthermore, sequences aligned to the Clostridia were also detected in higher abundances in the mucous samples, albeit only slightly. Members of this class of anaerobes are frequently recorded in the gut of terrestrial animals and humans, but were present in low sequence abundances in this study. Clostridia have been reported to dominate the hindgut of herbivorous fish, suggesting that their presence may correlate with host dietary habits and gut physiology (Mountfort et al 2002, Clements et al 2007, 2014). These data suggest that the mucosal layer may be a favourable niche for obligate anaerobes in the rainbow trout intestine, but that many bacterial species with this type of metabolism, with the exception of *Cetobacterium* in this case, are present in lower numbers in this species than those recorded in herbivorous fish, terrestrial animals and humans.

Cetobacteria have previously been isolated in both culture dependant and molecular based studies and this anaerobe frequently dominates molecular microbial libraries from the freshwater fish intestinal tract (Rawls et al 2006, Kim et al 2007, Wu et al 2010, van Kessel et al 2011, Di Maiuta et al 2013, Xia et al 2014). Whilst the total number of Fusobacteria sequences was higher in the lumen, sequences identified as *Cetobacterium* were slightly higher in the mucous. Indeed, this genus was the most abundant organism recorded in the intestinal mucous samples. Kim et al (2007) reported the presence of this bacterium in the rainbow trout

intestinal lumen using a clone-library based molecular analysis, and Tsuchiya et al (2008) described the isolation and characterization of *C. somerae* from the GI tract of five freshwater fish, including rainbow trout and carp. Furthermore, Rawls et al (2006) showed this bacterium to be present in the gut of zebrafish. The potential role of *C. somerae* in the GI tract of fish has not however been comprehensively explored. In humans, *Cetobacterium* spp. isolated from human faeces were shown to ferment peptides and carbohydrates (Finegold et al 2003) while Tsuchiya et al (2008) described the production of vitamin B12 by *C. somerae* isolated from the GI tract of freshwater fish. The combination of a fermentative metabolism and vitamin production indicates that this microbe could play a key role in the process of digestion in fish. The number of sequences attributed to this genus across each individual fish sampled in this study is a clear indication of this possibility.

The data presented in this study form at least part of a 'microbial map' of the rainbow trout intestine, and provide evidence of the presence of a very diverse microbial community present in both of the regions studied. The microbial diversity of both regions was more complex than much of the literature has described, suggesting that these niches, and in particular the mucosa, require further exploration with NGS-based approaches. It is believed that the microbial composition of the fish intestine may vary with age, nutritional status, environmental conditions, genotype, morphology of the different regions of the GI tract, and between individual fish (Cahill 1990, Ringø et al 1995, 2003, Xia et al 2014). This serves to underline the complexity of this ecosystem. This study has however attempted to address the level of variability between the microbial communities in the distal intestine of each individual fish, using NGS to circumvent the need to pool samples, and therefore present a more accurate semi-quantitative description of the true microbial ecology of the distal intestine. Many of these microbes may play key roles in the functioning of a healthy gut in these fish, and thus could potentially represent biomarkers of a healthy host. Moreover, a large number of these bacteria are not yet documented in ribosomal sequence databases, and their roles are yet to be identified. Research is underway to determine the functional role of these microbial organisms in the fish gut, and to investigate how these potential roles relate to improved fish health status and growth performance.

2.6. Acknowledgements

2.7. Conflicts of interest

The authors declare no conflict of interest.

2.8. References

Andersson, A.F., Lindberg, M., Jakobsson, H., Backhed, F., Nyren, P. and Engstrand, L. (2008) Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLOS ONE* **3**, e2836

Austin, B. and Al-Zahrani, A.M.J. (1988) The effect of antimicrobial compounds on the gastrointestinal microflora of rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **33**, 1-14

Austin, B. (2006) The bacterial microflora of fish, revised. *The Scientific World Journal* **6**, 931-945

Austin, B. and D.A. Austin. (1987) *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*. Ellis Horwood, Chichester, UK

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. and Gordon, J.I. (2005) Host-bacterial mutualism in the human intestine. *Science* **307**, 1915-1920

Backhed, F., Fraser, C., Ringel, Y., Sanders, M., Sartor, R., Sherman, P., Versalovic, J., Young, V. and Finlay, B. (2012) Defining a healthy human gut microbiome: Current concepts, future directions, and clinical applications. *Cell Host & Microbe* **12**, 611-622

Balcazar, J.L., De Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Girones, O. and Muzquiz, J.L. (2007) Sequencing of variable regions of the 16S rRNA gene for identification of lactic acid bacteria isolated from the intestinal microbiota of healthy salmonids. *Comparative Immunology, Microbiology and Infectious Diseases* **30**, 111-118

Balcazar, J.L., Vendrell, D., De Blas, I., Ruiz-Zarzuela, I., Muzquiz, J.L. and Girones, O. (2008) Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture* **278**, 188-191

Bolnick, D.I., Snowberg, L.K., Hirsch, P.E., Lauber, C.L., Knight, R., Caporaso, J.G. and Svanback, R. (2014) Individuals' diet diversity influences gut microbial diversity in two freshwater fish (threespine stickleback and Eurasian perch). *Ecology Letters* **17**, 979-987

Cahill, M.M. (1990). Bacterial flora of fishes: A review. *Microbial Ecology* **19**, 21-41

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Feirer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G. and Knight, R. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal* **6**, 1621-1624

Carda-Dieguez, M., Mira, A. and Fouz, B. (2014) Pyrosequencing survey of intestinal microbiota diversity in cultured sea bass (*Dicentrarchus labrax*) fed functional diets. *FEMS Microbiology Ecology* **87**, 451-459

Chen, P.E., Cook, C., Stewart, A.C., Nagarajan, N., Sommer, D.D., Pop, M., Thomason, B., Lentz, S., Nolan, N., Sozhamannan, S., Sulakvelidze, A., Mateczun, A., Du, L., Zwick, M.E. and Read, T.D. (2010) Genomic characterization of the *Yersinia* genus. *Genome Biology* **11:** R1-2010-11-1-r1

Clements, K., Pasch, I., Moran, D. and Turner, S. (2007) Clostridia dominate the 16S rRNA gene libraries prepared from the hindgut of temperate marine herbivorous fishes. *Marine Biology* **150**, 1431-1440

Clements, K.D., Angert, E.R., Montgomery, W.L. and Choat, J.H. (2014) Intestinal microbiota in fishes: what's known and what's not. *Molecular Ecology* **23**, 1891-1898.

Desai, A.R., Links, M.G., Collins, S.A., Mansfield, G.S., Drew, M.D., Van Kessel, A.G. and Hill, J.E. (2012) Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **350**, 134-142

Di Maiuta, N., Schwarzentruber, P., Schenker, M. and Schoelkopf, J. (2013) Microbial population dynamics in the faeces of wood-eating loricariid catfishes. *Letters in Applied Microbiology* **56**, 401-407

Durban, A., Abellan, J.J., Jiminez-Hernandez, N., Ponce, M., Ponce, J., Sala, T., D'Auria, G., Latorre, A. and Moya, A. (2011) Assessing gut microbial diversity from feces and rectal mucosa. *Microbial Ecology* **61**, 123-133

Finegold, S.M., Vaisanen, M.L., Molitoris, D.R., Tomzynski, T.J., Song, Y., Liu, C., Collins, M.D. and Lawson, P.A. (2003) *Cetobacterium somerae* sp. nov. from human feces and emended description of the genus *Cetobacterium*. *Systematic and Applied Microbiology* **26**, 177-181

Floris, R., Manca, S. and Fois, N. (2013) Microbial ecology of intestinal tract of gilthead sea bream (*Spaurus aurata* Linnaeus, 1758) from two coastal lagoons of Sardinia (Italy). *Transitional Waters Bulletin* **7**, 4-12

Franchini, P., Fruciano, C., Frickey, T., Jones, J.C. and Meyer, A. (2014) The gut microbial community of Midas cichlid fish in repeatedly evolved limnetic-benthic species pairs. *PLOS ONE* **9**, e95027

Guarner, F. and Malagelada, JR. (2003) Gut flora in health and disease. *Lancet* **361**, 512-519

Hartviksen. M., Vecino, J.L.G., Ringø, E., Bakke, A., Wadsworth, S., Krogdahl, Å., Ruohonen, K. and Kettunen, A. (2014) Alternative dietary protein sources for Atlantic salmon (*Salmo salar* L.) effect on intestinal microbiota, intestinal and liver histology and growth. *Aquaculture Nutrition* **20**, 381-398

Heikkinen, J., Vielma, J., Kemilainen, O., Tiirola, M., Eskelinen, P., Kiuru, T., Navia-Paldanius, D. and Von Wright, A. (2006) Effects of soybean meal based diet on growth performance, gut histopathology and intestinal microbiota of juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **261**, 259-268

Holben, W.E., Williams, P., Gilbert, M., Saarinen, M., Sarkilahti, L.K. and Apajalahti, J.H. (2002) Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. *Microbial Ecology* **44**, 175-185

Homlberg, S.D. and Farmer, J.J. (1984) *Aeromonas hydrophila* and *Plesiomonas shigelloides* as causes of intestinal infections. *Clinical Infectious Diseases* **6**, 633-639

Hovda, M.B., Lunestad, B.T., Fontanillas, R. and Rosnes, J.T. (2007) Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture* **272**, 581-588

Hoyles, L., Lawson, P.A., Foster, G., Falsen, E., Ohlen, M., Frainger, J.M. and Collins, M.D. (2000) *Vagococcus fessus* sp. nov., isolated from a seal and a harbour porpoise. *International Journal of Systematic Evolutionary Microbiology* **50**, 1151-1154

Huber, I., Spanggaard, B., Appel, K.F., Rossen, L., Nielsen, T. and Gram, L. (2004) Phylogenetic analysis and in situ identification of the intestinal microbial community of rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Journal of Applied Microbiology* **96**, 117-132

Ingerslev, H., von Gersdorff Jørgensen, L., Lenz Strube, M., Larsen, N., Dalsgaard, I., Boye, M. and Madsen, L. (2014a) The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. *Aquaculture* **424**, 24-34

Ingerslev, H.C., Strube, M.L., Jørgensen, L., Dalsgaard, I., Boye, M. and Madsen, L. (2014b) Diet type dictates the gut microbiota and the immune response against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology* **40**, 624-633

Kim, D.H., Brunt, J. and Austin, B. (2007) Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). *Journal of Applied Microbiology* **102**, 1654-1664

Kim, D.H. and Austin, B. (2008) Characterization of probiotic carnobacteria isolated from rainbow trout (*Oncorhynchus mykiss*) intestine. *Letters in Applied Microbiology* **47**, 141-147

Langille, M.G., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Vega Thurber, R.L., Knight, R., Beiko, R.G. and Huttenhower, C. (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology* **31**, 814-821

Lawson, P.A., Foster, G., Falsen, E., Ohlen, M. and Collins, M.D. (1999) *Vagococcus lutrae* sp. nov., isolated from the common otter (*Lutra lutra*). *International Journal of Systematic Bacteriology* **49**, 1251-1254

Li, W. and Godzik, A. (2006) Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* **22**, 1658-1659

Li, T.L., Mazeas, L., Sghir, A., leblon, G. and Bouchez, T. (2009) Insights into networks of functional microbes catalyzing methanization of cellulose under mesophilic conditions. *Environmental Microbiology* **11**, 889-904

Li, H., Zhong, Q., Wirth, S., Wang, W., Hao, Y., Wu, S., Zou, H., Li, W. and Wang, G., (2014) Diversity of autochthonous bacterial communities in the intestinal mucosa of grass carp (*Ctenopharyngodon idellus*) (Valenciennes) determined by culture-dependent and culture-independent techniques. *Aquaculture Research* **46**, 2344-2359

Magoc, T. and Salzberg, S.L. (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27**, 2957-2963

Manichanh, C., Reeder, J., Gibert, P., Varela, E., Llopsis, M., Antolin, M., Guigo, R., Knight, R. and Guarner, F. (2010) Reshaping the gut microbiome with bacterial transplantation and antibiotic intake. *Genome Research* **20**, 1411-1419

Merrifield, D.L., Burnard, D., Bradley, G., Davies, S.J. and Baker, R.T.M. (2009) Microbial community diversity associated with the intestinal mucosa of farmed rainbow trout (*Oncorhynchus mykiss* Walbaum). *Aquaculture Research* **40**, 1064-1072

Merrifield, D.L., Dimitroglou, A., Bradley, G., Baker, R.T.M. and Davies, S.J. (2010a) Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) I. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria. *Aquaculture Nutrition* **16**, 504-510

Merrifield, D.L., Bradley, G., Baker, R.T.M. and Davies, S.J. (2010b) Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) II. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria postantibiotic treatment. *Aquaculture Nutrition,* **16**, 496-503

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bogwald, J., Castex, M. and Ringø, E. (2010c) The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* **302**, 1-18

Moosvi, S.A., Pacheco, C.C., McDonald, I.R., De Marco, P., Pearce, D.A., Kelly, D.P. and Wood, A.P. (2005) Isolation and properties of methanesulfonate-degrading *Afipia felis* from Antarctica and comparison with other strains of *A. felis*. *Environmental Microbiology* **7**, 22-33

Morgan, X.C. and Huttenhower, C. (2012). Human microbiome analysis. *PLOS Computational Biology* **8**, e1002808

Mountfort, D.O., Campbell, J. and Clements, K.D. (2002) Hindgut fermentation in three species of marine herbivorous fish. *Applied and Environmental Microbiology* **68**, 1374-1380

Mulder, I.E., Schmidt, B., Stokes, C.R., Lewis, M., Baily, M., Aminov, R.I., Prosser, J.I., Gill, B.P., Pluske, J.R., Mayer, C.D., Musk, C.C. and Kelly, D. (2009) Environmentally-acquired bacteria influence microbial diversity and natural innate immune responses at gut surfaces. *BMC Biology* **7**, 79 **doi**: 10.1186/1741-7007-7-79

Nakayama, J., Jiang, J., Watanabe, K., Chen, K., Ninxin, H., Matsuda, K., Kurakawa, T., Tsuji, H., Sonomoto, K. and Lee, Y.K. (2013) Up to species-level community analysis of human gut microbiota by 16S rRNA amplicon pyrosequencing. *Bioscience of Microbiota, Food and Health* **32**, 69-76

Nam, Y.D., Jung, M.J., Roh, S.W., Kim, M.S. and Bae, J.W. (2011) Comparative analysis of Korean human gut microbiota by barcoded pyrosequencing. *PLOS ONE* **6**, e22109

Navarrete, P., Espejo, R.T. and Romero, J. (2009) Molecular analysis of microbiota along the digestive tract of juvenile Atlantic salmon (*Salmo salar* L.). *Microbial Ecology* **57**, 550-561

Navarrete, P., Magne, F., Mardones, P., Riveros, M., Opazo, R., Suau, A., Pochart, P. and Romero, J. (2010) Molecular analysis of intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*). *FEMS Microbiology Ecology* **71**, 148-156

Navarrete, P., Magne, F., Araneda, C., Fuentes, P., Barros, L., Opazo, R., Espejo, R. and Romero, J. (2012) PCR-TTGE analysis of 16S rRNA from rainbow trout (*Oncorhynchus*

mykiss) gut microbiota reveals host-specific communities of active bacteria. *PLOS ONE* **7**, e31335

Nayak, S.K. (2010) Role of gastrointestinal microbiota in fish. *Aquaculture Research* **41**, 1553-1573

Olsen, R., Sundell, K., Hansen, T., Hemre, G., Myklebust, R., Mayhew, R. and Ringo, E. (2002) Acute stress alters the intestinal lining of Atlantic salmon, *Salmo salar* L.: An electron microscopical study. *Fish Physiology and Biochemistry* **26,** 211-221

Parks, B.W., Nam, E., Org, E., Kostem, E., Norheim, F., Hui, S.T., Pan, C., Civelek, M., Rau, C.D., Bennett, B.J., Mehrabian, M., Ursell, L.K., He, A., Castellani, L.W., Zinker, B., Kirby, M., Drake, T.A., Drevon, C.A., Knight, R., Gargalovic, P., Kirchgessner, T., Eskin, E. and Lusis, A.J. (2013) Genetic control of obesity and gut microbiota composition in response to high-fat, high-sucrose diet in mice. *Cell Metabolism* **17**, 141-152

Pond, M.J., Stone, D.M. and Alderman, D.J. (2006) Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **261**, 194-203

Rawls, J.F., Mahowald, M.A., Ley, R.E. and Gordon, J.I. (2006) Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell* **127**, 423-433

Ringø, E., Strom, E. and Tabachek, J. (1995) Intestinal microflora of salmonids: a review. *Aquaculture Research* **26**, 773-789

Ringø, E. and Gatesoupe, F. (1998) Lactic acid bacteria in fish: a review. *Aquaculture* **160**, 177-203

Ringø, E., and T.H. Birbeck. (1999) Intestinal microflora of fish larvae and fry. *Aquaculture Research* **30**, 73-93

Ringø, E., Olsen, R.E., Mayhew, T.M. and Myklebust, R. (2003) Electron microscopy of the intestinal microflora of fish. *Aquaculture* **227**, 395-415

Ringø, E., Myklebust, R., Mayhew, T.M. and Olsen, R.E. (2007) Bacterial translocation and pathogenesis in the digestive tract of larvae and fry. *Aquaculture* **268**, 251-264

Ringø, E. (2008) The ability of carnobacteria isolated from fish intestine to inhibit growth of fish pathogenic bacteria: a screening study. *Aquaculture Research* **39**, 171-180

Roeselers, G., Mittge, E., Stephens, W., Parichy, D., Cavanaugh, C., Guillemin, K. and Rawls, JF. (2011) Evidence for a core gut microbiota in the zebrafish. *The ISME Journal* **5**, 1595-1608

Salinas, I., Myklebust, R., Esteban, M.A., Olsen, R.E., Meseguer, J. and Ringø, E. (2008) In vitro studies of *Lactobacillus delbrueckii* subsp. *lactis* in Atlantic salmon (*Salmo salar* L.) foregut: tissue responses and evidence of protection against *Aeromonas salmonicida* subsp. *salmonicida* epithelial damage. *Veterinary Microbiology* **128**, 167-177

Schloss, P.D. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**, 7537-7541

Skrodenyte-Arbaciauskiene, V., Sruoga, A. and Butkauskas, D. (2006) Assessment of microbial diversity in the river trout *Salmo trutta fario* L. intestinal tract identified by partial 16S rRNA sequence analysis. *Fisheries Science* **72**, 597-602

Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T., Appel, K.F. and Gram, L. (2000) The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. *Aquaculture* **182**, 1-15

Sugita, H., Miyajima, C. and Deguchi, Y. (1991) The vitamin B12 producing ability of the intestinal microflora of freshwater fish. *Aquaculture* **92**, 267-276

Tamer, A.U., Aragno, M. and Sahin, N. (2002) Isolation and characterization of a new type of aerobic, oxalic acid utilizing bacteria, and proposal of *Oxalicibacterium flavum* gen. nov., sp. nov. *Systematic and Applied Microbiology* **25**, 513-519

Tapia-Panigua, S.T., Reyes-Berrecil, M., Ascencio-Valle, F., Esteban, M.A., Clavijo, E., Balebona, M.C. and Morinigo, M.A. (2011) Modulation of the intestinal microbiota and immune system of farmed *Spaurus aurata* by the administration of the yeast *Debaryomyces*

hansenii L2 in conjunction with inulin. *Journal of Aquaculture Research and Development* **S1**: 012, **doi**: 10.4172/2155-9546.S1-012

Teixeria, L.M., Carvalho, M.G., Merquior, V.L., Steigerwalt, A.G., Brenner, D.J. and Facklam, R.R. (1997) Phenotypic and genotypic characterization of Vagococcus fluvialis, including strains isolated from human sources. *Journal of Clinical Microbiology* **35**, 2778-2781

Tsuchiya, C., Sakata, T. and Sugita, H. (2008) Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. *Letters in Applied Microbiology* **46**, 43-48

Turnbaugh, P., Ley, R., Mahowald, M., Magrini, V., Mardis, E. and Gordon, J. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027-1031

Turnbaugh, P.J. and Gordon, J.I. (2009) The core gut microbiome, energy balance and obesity. *The Journal of Physiology* **587**, 4153-4158

Upadrasta, A., O'Sullivan, L., O'Sullivan, O., Sexton, N., Lawlor, P.G., Hill, C., Fitzgerald, G.F., Stanton, C. and Ross, R.P. (2013) The effect of dietary supplementation with spent cider yeast on the swine distal gut microbiome. *PLOS ONE* **8**, e75714

van Kessel, M., Dutilh, B., Neveling, K., Kwint, M., Veltman, J., Flik, G., Jetten, M., Klaren, P. and Op den Camp, H. (2011) Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (*Cyprinus carpio* L.) *AMB Express* **1**:41, **doi:** 10.1186/2191-0855-1-41

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T., Wiens, G.D., Snekvik, K., Rawls, J.F. and Good, C. (2013) Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. *Applied and Environmental Microbiology* **79**, 4974-4984

Wu, S., Gao, T., Zheng, Y., Wang, W., Cheng, Y. and Wang, G. (2010) Microbial diversity of intestinal contents and mucus in yellow catfish (*Pelteobagrus fulvidraco*). *Aquaculture* **303**, 1-7

Xia, J., Lin, G., Fu, G., Wan, Z., Lee, M., Wang, L., Liu, X. and Yue, G. (2014) The intestinal microbiome of fish under starvation. *BMC Genomics* **15**:266, **doi**: 10.1186/1471-2164-15-266

Zarate, G., Gonzalez, S. and Chaia, A.P. (2004) Assessing survival of dairy propionibacteria in gastrointestinal conditions and adherence to intestinal epithelia. *Methods in Molecular Biology (Clifton, N.J.)* **268**, 423-432

Zarkasi, K., Abell, G., Taylor, R., Neuman, C., Hatje, E., Tamplin, M., Katouli, M. and Bowman, J.P. (2014) Pyrosequencing based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system. *Journal of Applied Microbiology* **117**, 18-27

2.9. Supplementary information

Table 2.4. (S1). **Niche and potential function of abundant bacterial genera identified through 16S rRNA amplicon sequencing from rainbow trout GI tract.**

Genus	Phylum	Metabolism	Niche and Function	Reference
Cetobacterium	Fusobacteria	Obligate anaerobes	Recorded in faeces of rainbow trout and catfish, vitamin B12 production.	Sugita et al 1991, Wu et al 2010, Tsuchiya et al 2008, Di Maiuta et al 2013
Bacilli	Firmicutes	Aerobic heterotrophs	Known members of the terrestrial animal GI tract, recorded in fish intestine, fermentative.	Ringø & Gatesoupe 1998
Porphyromonadaceae	Bacteroidetes	Obligate anaerobes	Some species pathogenic, omnipresent in human GI tract, also present in fish intestines, fermentative.	Mulder et al 2009, Wu et al 2010, Li et al 2009
Carnobacterium	Firmicutes	Facultative anaerobes	Some species pathogenic, normal intestinal flora of healthy fish, fermentative.	Ringø & Gatesoupe 1998, Kim & Austin 2008, Kim et al 2007
Obesumbacterium	γ Proteobacteria	Facultative anaerobes	Occurs as a brewery contaminant, also part of microflora of terrestrial animals, rarely recorded as present in fish intestines. Fermentative.	Navarrete et al 2009, 2012, Skrondenyte-Arbaciauskiene et al 2006
Hafnia	γ Proteobacteria	Facultative anaerobes	Fermentative, with production of acid and gas. Opportunistic pathogen for humans and fish. Occurs as a commensal in GI tract of fish, terrestrial animals and humans.	
Enterococcus	Firmicutes	Facultative anaerobes	Fermentative, production of lactic acid. Common commensal organisms in GI tract of humans, also recorded in fish intestines.	Ringø & Gatesoupe 1998
Plesiomonas	γ Proteobacteria	Facultative anaerobes	Fermentative, with production of acid. Occurs naturally in freshwater, and in intestines of fish and terrestrial animals, opportunistic pathogen.	Holmberg & Farmer 1984, Austin 2006
Serratia	γ Proteobacteria	Facultative anaerobes	Occurs naturally in the environment in soil and fresh water. Colonizes respiratory and urinary tract of humans and is responsible for nosocomial infections. Commonly found in GI tract of rodents, insects and fish.	Tapia-Paniagua et al 2011

Staphylococcus	Firmicutes	Facultative anaerobes	Respiratory and fermentative. Mainly associated with the skin and mucous membranes of warm blooded vertebrates. Recorded in the GI tract of fish.	Ringø & Gatesoupe 1998, Wong et al 2013
Pseudomonas	γ Proteobacteria	Aerobes	Respiratory, but also capable of anaerobic growth. Widely distributed in nature. Some species pathogenic for humans, animals, plants and fish. Frequently recorded in GI tract of fish.	Di Maiuta et al 2013, Merrifield et al 2009, Ringø et al 1995
Bradyrhizobium	β Proteobacteria	Aerobic	Chemoorganotrophic. Present in soil, nitrogen fixing. Recorded in fish intestinal epithelium.	Carda-Dieguez et al 2014
Ralstonia	β Proteobacteria	Facultative anaerobes	Ubiquitous in water and soil. Has been documented in yellow catfish and rainbow trout. Denitrification of nitrate and/or nitite to nitrogen gas.	Kim et al 2007, Wu et al 2010
Streptococcus	Firmicutes	Facultative anaerobes	Fermentative, producing lactate but no gas. Inhabit the mouth and upper respiratory tract in vertebrates. Recorded in fish GI tract. Some species pathogenic.	Ringø & Gatesoupe 1998
Oxalicibacterium	β Proteobacteria	Aerobic	Utilizes oxalic acid. Found in feces of chickens, suggesting that it is present in GI tract. Strains reported as present in soil.	Tamer et al 2002
Vagococcus	Firmicutes	Facultative anaerobes	Chemoorganotrophic, fermentative. Isolated from terrestrial animal faeces, human clinical specimens, diseased harbour seals and porpoises. Present in intestines of healthy salmon and trout, but some strains may be pathogenic.	Teixeira et al 1997, Ringø & Gatesoupe 1998, Lawson et al 1999, Hoyles et al 2000
Enterococcus	Firmicutes	Facultative anaerobes	Fermentative. Occur widely in the environment, particularly in feces of vertebrates. Also recorded in GI tract of fish.	Ringø & Gatesoupe 1998
Flavobacterium	Bacteroidetes	Aerobic	Respiratory.Chemoorganotrophic. Widely distributed in soil and water, also found in raw meats, milk and other foods. Some species pathogenic to fish.	
Afipia	α Proteobacteria	Aerobic	Non-fermentative. *A. felis* has been associated with cat-scratch disease in humans. Recorded in sediment in estuarine environment, and may be free living and widespread.	Moosvi et al 2005
Escherichia	γ Proteobacteria	Facultative anaerobes	Motile by peritrichous flagella or are non-motile. Fermentative.	

			Occur as normal flora in the lower intestine of warm blooded animals, and also insects and fish.	
Lacticigenium	Firmicutes	Facultative anaerobes	Fermentative. Lactic acid major end product of glucose fermentation. Recorded in water surrounding crude oil well. Not recorded in fish GI tract to author's knowledge.	
Arthrobacter	Aerobic	Actinobacteria	Oxidative metabolism. Little or no acid and no gas produced from glucose and other carbohydrates. Widely distributed in the environment, principally in soil. Rarely recorded in fish GI tract.	
Corynebacterium	Actinobacteria	Facultative anaerobes	Fermentative. Primarily found in mucous membranes and skin of mammals. Recorded in GI tract of fish.	
Acinetobacter	γ Proteobacteria	Aerobic	Strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Occur naturally in soil, water and sewage.	
Cronobacter	γ Proteobacteria	Facultative anaerobes	Fermentative, and produce acid. Causes disease in humans, particularly infants. Has been found in food and often in sewage water.	
Acidovorax	β Proteobacteria	Aerobic	Oxidative carbohydrate metabolism, with oxygen as the terminal electron acceptor. Widespread in soil and water.	
Comamonas	β Proteobacteria	Aerobic	Non fermentative and chemoorganotrophic. Regularly found/used to treat wastewater in sewage plants. Also found in soil. Forms flocs due to presence of a mucopolysaccharide.	
Herminiimonas	β Proteobacteria	Facultative anaerobes	Ultramicrobacteria. Reduce nitrate to nitrite. Different species have been recorded in spring and sewage waters, as well as in arctic glacial ice samples.	
Pantoea	γ Proteobacteria	Facultative anaerobes	Fermentative. Form mucoid colonies on agar. Isolated from environment (plant surfaces, water, soil). Some species are opportunistic human pathogens. Recorded in intestinal mucous samples from rainbow trout.	Kim et al 2007

Chapter 3

Effects of low-level dietary microalgae supplementation on the distal intestinal microbiome of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum)

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3.1. Abstract

In this study, high throughput 16S rRNA sequencing was used to investigate the effect of a novel whole-cell dietary microalgae meal (*Schizochytrium limacinum*), on the distal intestinal microbiome of farmed rainbow trout *Oncorhynchus mykiss*. Heterotrophic microalgae are rich in omega 3 polyunsaturated fatty acids, can be produced sustainably, and have been shown to have beneficial effects on host health. After a 15-week trial period, microbial community profiles were compared between the distal intestinal contents of fish fed either a control diet or a treatment diet that partially replaced fish oil with microalgae meal at a rate of 20%, and which represented 5% of the overall feed formulation. The results of this research showed that the microbial communities of both fish populations were composed of similar microbial taxa, however the treatment group fed the microalgae supplement possessed a greater level of microbial diversity than those in the control group. A limited number of bacterial taxa were discriminatory between diets and were significantly elevated in the treatment group, notably OTU's assigned to the genera *Streptococcus*, *Leuconostoc*, *Lactobacillus, Lactococcus* and *Weissella*. However, the overall structure of the intestinal microbiome between control and treatment groups was not found to be significantly different. The treatment group displayed a heavier mean weight and condition factor at the end of the trial period. The results of this study suggest that the tested microalgae meal can be used as a replacement for a proportion of fish oil in aquafeeds, with minor changes to the intestinal microbiome of farmed rainbow trout, and positive effects on growth.

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3.2. Introduction

Numerous studies have reported that diet type is a major driver in shaping the bacterial communities of the GI tract, commonly referred to as the 'microbiome', of both terrestrial and aquatic animals (Ringø & Olsen 1999, Merrifield et al 2010, 2011, Gatesoupe et al 2014, Kormas et al 2014, Miyake et al 2015). These microbes are believed to play important roles in host development, immunity, digestion and nutrition (Romero et al 2014). The impacts of novel dietary ingredients on the intestinal microbiome of farmed salmonids, in particular those of probiotics, prebiotics and immunostimulants, are receiving more attention as aquafeed formulations evolve in line with restrictions on antibiotic use and the diminishing availability of marine fishmeal and fish oil (Tacon & Metian 2008). The vast majority of these investigations have thus far been undertaken using culture-based and low resolution molecular microbiological techniques that can provide only a partial description of the composition of the teleost intestinal microbiome and its potential response to dietary manipulation (Spanggaard et al 2000, Nayak 2010).

However, more recently, high throughput sequencing technologies have been used to examine the effect of diet on the intestinal microbiome of fish in far greater detail. Desai et al (2012) used 454 pyrosequencing to demonstrate reproducible alterations of the intestinal microbiome of farmed rainbow trout fed soybean meal (SBM), noting changes in the ratio of Firmicutes:Proteobacteria as a result of supplementation. In two further studies, Ingerslev et al (2014a, b) used the Illumina HiSeq® platform to demonstrate changes in the structure of the intestinal microbiome of rainbow trout fry fed either marine or plant-based dietary ingredients. In contrast to these results, Wong et al (2013) reported that the intestinal microbiome of rainbow trout is largely unaffected by dietary alterations and observed only very minor changes among specific microbial community assemblages. Furthermore, substantial inter-animal variation has been noted in the structure of the intestinal microbiome in individual fish, (Mansfield et al 2010) suggesting that analyses of pooled samples are unsuitable in studies of

the intestinal microbiome. HTS platforms play an important role in this regard, in that they permit high resolution analyses of individual gut microbiomes, leading to more reliable conclusions regarding the effect of dietary alterations on the structure of the microbial communities within the fish GI tract.

It has been widely reported that the gut microbiome of aquatic animals is responsible for the digestion of algal cells and the production of both amino acids and short-chain fatty acids, in addition to the secretion of inhibitory compounds that can suppress the growth of potential pathogens (Austin 2006, Nayak 2010, Clements et al 2014, Ghanbari et al 2015). Research concerning the impact of microalgae on the structure of the intestinal microbiome however is limited and has hitherto primarily focused on wild herbivorous fish species that consume algal substrates in their natural habitat (Choat & Clements 1998, Clements et al 2007, Ward et al 2009, Smriga et al 2010) with only a single study examining farmed fish species (Cerezuela et al 2012). Conflicting results have been reported in these studies, with some authors observing increases and others noting decreases in microbial diversity within the intestinal tracts of fish species with diets rich in microalgae. This suggests that whilst diet impacts the bacterial diversity of the fish GI tract, the relationship between novel dietary components such as microalgae, and the structure of the intestinal microbiome, is not clear and thus further detailed examination is undoubtedly required.

The primary objective of this study was to characterize the intestinal microbiome of farmed rainbow trout fed either a standard commercial diet, or a treatment diet containing 5% microalgae meal, in order to test whether minor differences in diet composition lead to alterations in the structure of the microbial community of this region. The aquafeed sector recognizes the need to provide dietary alternatives to fish oil, which provide comparative health benefits to farmed fish species. Therefore, the secondary aim of this research was to test for any differences in growth performance between the control and treatment groups and whether or not this could be correlated with the composition of the intestinal microbiome. It was hypothesized that feeding farmed rainbow trout slightly different diets would alter the structure of the intestinal microbiome in these fish.

3.3. Materials and methods

3.3.1. Dietary formulation

Two diets, one control and one treatment, were formulated at the Hellenic Centre for Marine Research (HCMR, Anavyssos Attiki, Greece). These diets were similar except that the experimental diet contained a whole cell microalgae ingredient (ALL-G-Rich™, *Schizochytrium limacinum*; Alltech Biotechnology Inc., Nicholasville USA) at an overall inclusion level corresponding to 5% of the total diet formulation (Table 3.1). Both diets met or exceeded the guideline nutrient requirements for rainbow trout (National Research Council 2011).

Table 3.1. Ingredient composition and nutrient analysis of diets

	Diet 1 (Control)	Diet 2 (Treatment)
Ingredient	**% Inclusion**	**% Inclusion**
Fish meal 68	22	22
Wheat meal	15	14
Wheat gluten	10	10
Soybean meal 47	14	14
Soybean concentrate 65	20	19
Alltech algae meal	0	5
Fish oil	15	12
Monocalcium phosphate	1.3	1.3
Mineral and vitamin premix	1	1
Lysine	0.6	0.6
Methionine	0.5	0.5
Dietary component		
Moisture	6.0	6.1
Protein	45.4	45.2
Fat	18.4	18.3
Ash	5.9	6.0
Fibre	1.1	1.1
NFE	20	19.2

3.3.2. Experimental design and sampling protocol

Farmed rainbow trout (*O. mykiss*) were obtained from a local trout farm and transferred to the Aquatic Research Facility (ARF) at the University of Stirling Institute of Aquaculture (Stirling, UK). The average weight of the fish on arrival at the ARF was 31.7 ± 2.6g. Fish were quarantined in a communal tank for 10 days, prior to random allocation into eight 100 L tanks (n=25 tank^{-1}) maintained on a flow through system, under a 12 h light and 12 h dark cycle and an ambient water temperature ($14 \pm 1^{\circ}$C). All instructions and guidelines set by the UK Home Office under the Animal Welfare Act of 1986 were adhered to throughout this experimental trial. Each tank was randomly allocated the diets, giving four replicates per treatment (Figure 3.1), and each group was hand fed a ration of approximately 2% of their body weight twice daily.

At the end of the 15-week trial period, a total of three fish from each of four replicates per treatment were randomly removed for sampling. Fish were sacrificed with a lethal dose of the anaesthetic benzocaine (Sigma Aldrich®, Dorset, UK) and swabbed with 100% ethanol before dissection through the ventral surface. The tissues surrounding the visceral fat were removed and the distal gut contents from a tissue section of approximately 2.5 cm in length (~150 mg) were aseptically collected by gently massaging the tissue with a sterile forceps, and placed into sterile 2 ml capped microtubes (Alpha laboratories®, Eastleigh, UK) containing 1 ml of lysis buffer (Qiagen®, Hilden, Germany). The gut was then incised and washed with a sterile 0.85% (w/v) NaCl solution, and the intestinal mucous was carefully removed from the gut wall. This material was placed into the same tube as the gut contents for each individual fish. All tubes were immediately placed on dry ice before DNA extraction later the same day, in order to ensure optimal sample integrity. In addition to the intestinal samples, three pellets from each diet and a sample of the tank biofilm were also processed as described above, to compare the microbial communities of both the diets themselves and of the tank biofilm, with the intestinal microbiome of the trout.

Figure 3.1. Experimental tanks at the Institute of Aquaculture, University of Stirling used for this trial. Tanks were randomly assigned either a ● control or ● treatment diet in a quadruplicate design.

3.3.3. Growth performance

The length and weight of each fish sampled at the end of the trial period was recorded to measure growth performance, thermal growth coefficient (TGC) and condition factor (K). Final fish weight was measured as the mean final weight of each group ± standard error of the mean (SEM). TGC was calculated using the formula TGC= $(W2^{(1/3)} - W1^{(1/3)})/D^{(0)} \times 1000$ where W2 and W1 are weight at the end and at the start of the trial respectively, and d^0 represents degree days. K was calculated using Fulton's equation K= $(10^5 \times weight)/Length^3$.

3.3.4. DNA extraction

A total of 150 mg of intestinal content material from each individual fish, suspended in 1 ml of buffer ASL (Qiagen), was processed for DNA extraction. A further sample containing only 1 ml of buffer ASL was processed as a negative control. Samples were firstly disrupted using a Mini bead-beater 16 (Biospec Ltd., Bartlesville, OK, USA) at maximum speed for four separate cycles of 35 s each. Samples were allowed to settle, and total genomic DNA was extracted and purified using the QIAamp DNA Stool Mini Kit (Qiagen), with the following modifications to the manufacturer's protocol: 150 mg starting material in 1 ml buffer ASL; suspension heated at 95°C for 10 min to improve lysis of Gram positive bacteria; 0.5 Inhibitex tablet per sample in 700 µl supernatant; final sample elution volume of 50 µl. After extraction, the DNA concentration of all samples was determined both spectrophotometrically (NanoDrop®1000, Thermo Scientific, Glasgow, UK) and fluorometrically (Qubit® Life Technologies, Paisley, UK) to ensure optimal DNA purity, and stored at -20°C for subsequent processing.

3.3.5. 16S rRNA PCR and Illumina sequencing

A PCR was first carried out using universal eubacterial primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Weisburg et al 1991) that target the full length bacterial 16S rRNA gene sequence, to confirm the presence of ample microbial community DNA and to rule out the presence of any potential inhibitory compounds. The extraction from buffer ASL was included in the PCR run to check for the presence of microbial DNA in the reagent itself. The PCR conditions for this confirmatory reaction were as follows; denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 2 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min; before final elongation at 72°C for 10 min. Products were then visualized on a 1.5% (w/v) agarose gel, run at 100V for approximately 1 h 15 min. The presence of a single strong PCR product of ~1500bp was considered to be indicative of the presence of microbial community DNA.

Illumina libraries were prepared following the method described by Caporaso et al (2012) using the NEXTflex 16S Amplicon-Seq kit (Bioo Scientific, Austin USA). A total of 12.5 ng of template DNA was used for each individual sample and the V4 hypervariable region of the bacterial 16S rRNA gene (length 292bp) was amplified using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (GATC Biotechnology Inc., Konstanz, Germany). The PCR conditions were as follows; initial denaturation at 95°C for 5 min; 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s; followed by a final extension step at 72°C for 5 min. All samples were amplified in triplicate and all products purified using Agencourt Ampure XP beads (Beckman Coulter Ltd.). The products of the first PCR served as template for a second PCR with the same conditions as the first, however the number of cycles was reduced to eight, and Illumina sequencing adapters were added to the primers in the reaction mix. Following amplification, PCR products were purified using Agencourt Ampure XP (Beckman Coulter, Wycombe, UK) with a modified 1:1 volume of PCR product to Ampure XP beads. Purified amplicons were quantified with Qubit®, pooled in equal concentration and the final quality of the pooled library was validated using a Bioanalyzer 2100® (Agilent Technologies, Waldbronn, Germany). The final library was prepared and sequenced using the Illumina MiSeq® NGS system at GATC Biotechnology (Konstanz, Germany).

3.3.6. Bioinformatics

Demultiplexing was performed with Casava v. 1.8 (Illumina) and reads representing the PhiX or reads not matching indices were removed. The open-source software mothur (Schloss 2009) was used to process sequences from the demultiplexed 16S rRNA gene libraries. Sequences were firstly merged using the make.contigs command. Reads containing ambiguous bases, homopolymer runs greater than 8 bases, and sequences of less than 250, or greater than 292, base pairs in length were removed from the dataset. Remaining sequences were aligned against mothur's Silva reference database, after customizing the reference alignment to concentrate on the V4 region only (length = 292bp). Further denoising of the dataset was performed using mothur's pre clustering algorithm, allowing for up to two differences between sequences. This sorted sequences by abundance, ordering from most abundant to least and identified sequences within two nucleotides of each other. If sequences met these conditions, they were merged. Chimeric sequences were then removed from the dataset using the UCHIME (Edgar et al 2011) algorithm in mothur as a final denoising step prior to taxonomic classification.

For taxonomic analyses, sequences were annotated using the Bayesian classifier implemented by the ribosomal database project (RDP) Release 11 (Centre for Microbial Ecology, Michigan State University, East Lansing, MI, USA). A minimum confidence bootstrap threshold of 80% was required for each assignment, thus >80% of the classifications returned the same taxonomic assignment for a given read, after one thousand iterations. Sample coverage, rarefaction curves, bias-corrected Chao 1 richness and Simpson's index of diversity were calculated based on assembled OTU's using mothur. Samples were rarefied to the sample with the lowest number of sequences before performing these diversity analyses, to ensure that any observed differences in diversity were not caused by uneven sampling depth.

3.3.7. Statistical analyses

A student's t-test was performed using Minitab 17 statistical software (www.mintab.com), to compare the growth performance data between control and treatment groups, and differences were considered significant at $p < 0.05$. The same software was also used to perform a one-way analysis of variance (ANOVA) test, using the Simpson and Chao 1 richness data, to determine whether the diversity of the intestinal microbiome was significantly different between both sample sets. Furthermore, the similarity of the structure and membership of the microbial communities found in each of the samples was calculated by creating a distance matrix based on the ThetaYC (Yue & Clayton 2005) coefficient using the dist.shared algorithm in mothur.

This distance matrix was visualized using principal coordinate analysis (PCoA), which allowed the intestinal microbial community profiles from the control and treatment groups to be compared. In addition, a dendrogram was created from these data using FigTree (Rambaut 2009) to further describe the similarity of the samples to each other. Parsimony (Schloss & Handelsman 2006) and UniFrac (Lozupone & Knight 2005) analyses were performed to determine whether any observed community structure clustering between diets was statistically significant. Finally, metatstats (White et al 2009), LEfSe (Segata et al 2011) and Indicator (McCune et al 2002) analyses were performed within mothur, in order to determine whether there were any phylotypes that exhibited a statistically significant representation between the control and treatment samples, and results were considered as significant at two levels, $p < 0.05$ and $p < 0.01$. The same statistical analyses were also used to compare feed pellet and tank biofilm samples with the intestinal samples from the control and treatment groups.

3.4. Results

3.4.1. Growth performance

All fish consumed both diets readily and upon conclusion of the trial, the weighed individuals from the treatment group had a higher mean weight and condition factor than the control group. The final mean weight and condition factor ($\pm$ SE) for the treatment group was 136.6 ± 12.1 g and 1.44 ± 0.06 whereas these values for the control group were 116.5 ± 9.3 g and 1.33 ± 0.04 respectively (Figure 3.2). However, the t-test found that there was no significant difference between the growth performance parameters for both groups ($p = 0.205$).
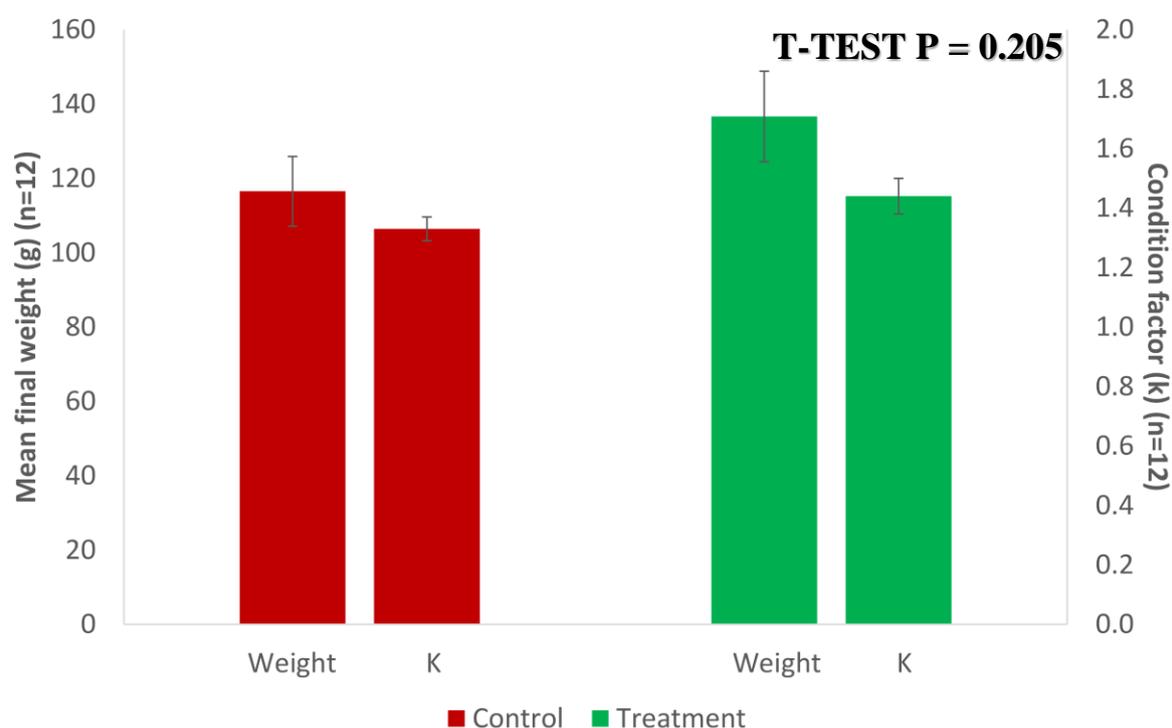
Figure 3.2. Growth performance data for control and treatment fish populations. Mean final weight (g) and condition factor (K) $\pm$ SEM at the end of the 15-week trial period are shown (n=12). Condition factor was calculated according to Fulton's method.

3.4.2. Sequence data and microbial diversity analyses

After quality filtering of sequences, a total of 18,282,541 sequences remained for analysis, which grouped into a total of 660 OTU's. After subsampling to that of the library containing the least number of reads (sample AF6, n=314,961), rarefaction curves generated in mothur showed a trend towards a greater level of microbial diversity in the treatment group with a greater number of overall OTU's being recorded (Figure 3.3). This trend was reflected in the inverse Simpson and Chao1 diversity indices, with the three richest samples (AF7, AF4 and AF6) belonging to the treatment group (Table 3.2). However, the overall level of microbial diversity was not found to be significantly different between both groups (Simpson: $f = 1.45$, $p = 0.241$; Chao 1: $f = 2.40$, $p = 0.136$) (Table 3.2, Figure 3.10 S2). A very high level of sequence coverage was achieved, with all rarefaction curves reaching saturation and Good's coverage estimations reaching >99% for each sample, indicating that the vast majority of microbial phylotypes present were sampled in the analysis.

Table 3.2. Alpha diversity estimates of rainbow trout intestinal microbiomes

			Simpson		Inverse Simpson		Chao 1	
Sample	OTU	Coverage	μ	σ	μ	σ	μ	σ
C F1	97	0.999917	0.64	0.000054	1.55	0.00013	157.35	2.43
C F2	73	0.999908	0.93	0.000026	1.07	0.00003	140.37	2.17
C F3	150	0.999908	0.35	0.000076	2.87	0.00062	206.02	1.88
C F4	90	0.999905	0.38	0.000125	2.63	0.00085	199.86	4.21
C F5	92	0.999917	0.84	0.000038	1.18	0.00005	178.19	4.03
C F6	58	0.999937	0.90	0.000031	1.10	0.00003	128.80	3.39
C F7	68	0.999914	0.94	0.000025	1.06	0.00003	159.93	4.29
C F8	62	0.999937	0.88	0.000033	1.13	0.00004	117.70	2.17
C F9	94	0.99993	0.89	0.000032	1.12	0.00004	143.69	1.96
C F10	173	0.999838	0.80	0.000017	1.25	0.00002	272.98	1.03
C F11	177	0.999882	0.67	0.000049	1.49	0.00010	237.94	1.89
C F12	62	0.999943	0.93	0.000028	1.07	0.00003	114.63	2.20
MeanC	99.6	0.999911	0.76	0.000045	1.46	0.00016	171.45	2.63
A F1	132	0.999835	0.49	0.000115	2.04	0.00047	284.33	4.73
A F2	87	0.999921	0.89	0.000031	1.12	0.00003	168.74	3.04
A F3	185	0.999851	0.65	0.000052	1.52	0.00012	248.07	1.74
A F4	228	0.999895	0.69	0.000051	1.45	0.00010	318.00	3.90
A F5	87	0.999902	0.85	0.000037	1.17	0.00005	182.45	3.47
A F6	192	0.99981	0.44	0.00004	2.28	0.00001	294.62	2.12
A F7	255	0.999816	0.56	0.000064	1.77	0.00020	332.80	1.93
A F8	77	0.999952	0.88	0.000034	1.13	0.00004	127.85	2.43
A F9	134	0.999886	0.59	0.00006	1.68	0.00017	219.07	2.77
A F10	80	0.999917	0.67	0.000052	1.49	0.00011	141.80	2.69
A F11	86	0.999975	0.38	0.000072	2.61	0.00048	103.96	1.01
A F12	83	0.999927	0.90	0.000033	1.11	0.00004	140.91	2.51
MeanA	135.5	0.999891	0.66	0.000053	1.61	0.00015	213.55	2.69

Normalized mean values (μ) and standard deviations (σ) for the number of OTU's, Sample coverage, Simpson Index, Inverse Simpson Index and Chao 1 richness. Normalized values were obtained by random resampling via rarefaction analysis according to the smallest sample size (n=314961, A F6) and standard errors were obtained by bootstrapping. OTU's are clustered according to a 97% sequence similarity cut-off value. Fish 1-12 are shown. C = Control samples, A= Treatment samples (Algae).

a)

b)
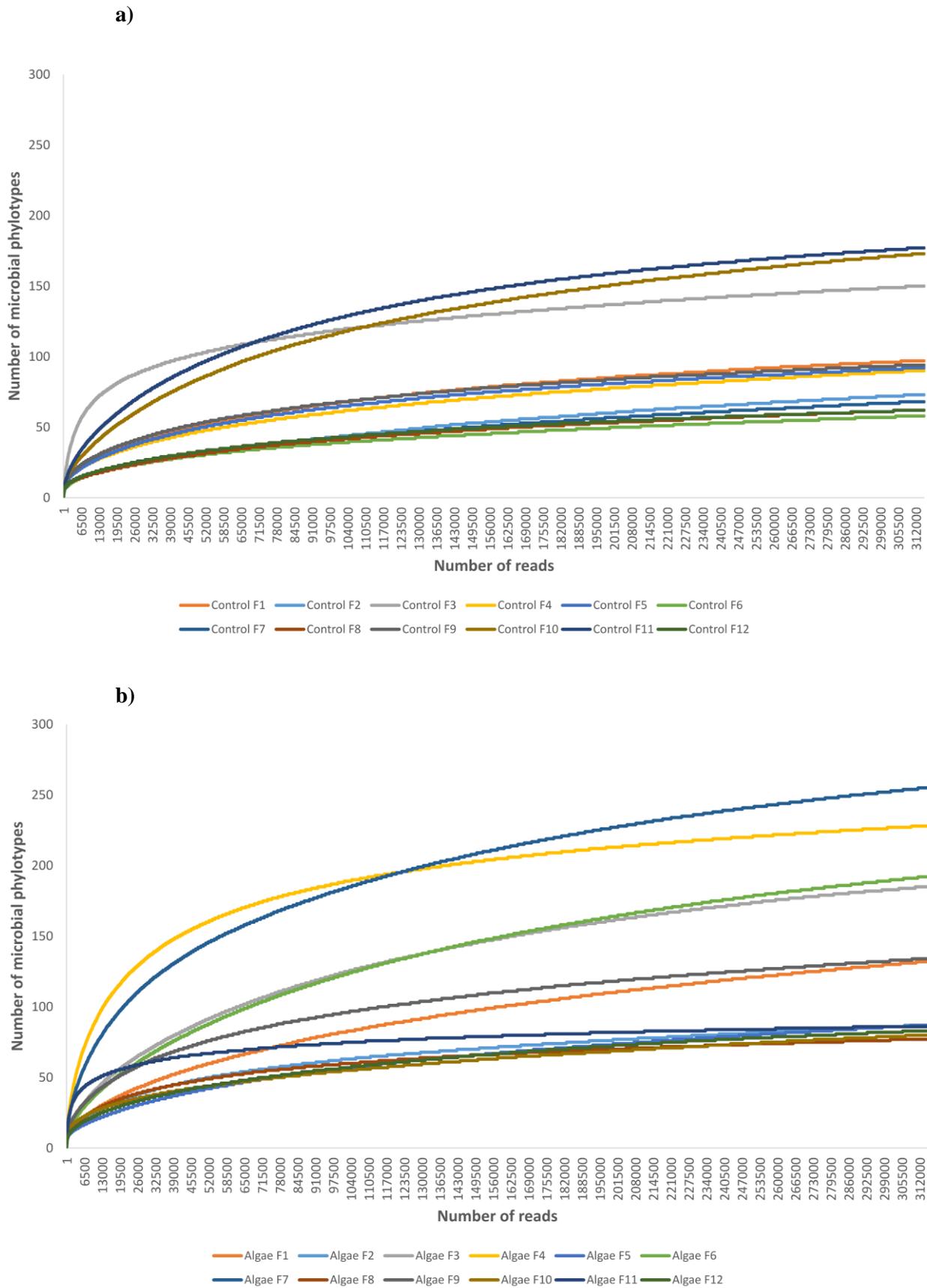
Figure 3.3. Rarefaction analysis of **a)** control and **b)** treatment group sequence libraries. Samples were rarefied according to the library with the lowest number of reads (n=314961, A F6).

3.4.3. Microbial community composition and influence of diets

The overall microbial community composition was similar in both the control and treatment populations of fish. The distribution of OTU's at the phylum level of both the control and treatment libraries is illustrated in Figure 3.4. The vast majority of reads were assigned to nine separate bacterial phyla, although an overall total of 13 phyla were recorded. Within these phyla, 13 microbial classes dominated, although 19 were recorded in total (Figure 3.5). The mean number of OTU's classified to genus level observed in the control group was 99 (maximum of 177, minimum of 58), whereas in the treatment group the mean was 135 (maximum of 255, minimum of 77) (Table 3.2), reflecting the trend towards an increased level of microbial diversity in these fish. Considerable variability amongst individuals was noted.

The Tenericutes were the dominant phylum identified in the libraries recovered from both the control and treatment groups, with *Mycoplasma* being the most dominant genus observed. This suggests that the abundance of *Mycoplasma* was not affected by diet type. The remaining OTU's primarily belonged to the Firmicutes, Proteobacteria and Spirochaetes. OTU's assigned to Bacteroidetes, Actinobacteria, Deinococcus-Thermus, Candidate Division WPS-1 and Fusobacteria were detected at much lower levels of sequence abundances. Within the Firmicutes, the most frequently observed OTU's were *Acetanerobacterium*, *Weissella*, *Catellicoccus*, *Streptococcus*, *Leuconostoc*, *Lactobacillus*, *Lactococcus*, *Ornithinibacillus* and *Sediminibacillus*. *Acetanaerobacterium* represented the second most dominant OTU recorded overall, and was present in higher mean relative sequence abundances in the group fed the control diet. Sequences assigned to the Proteobacteria were observed more frequently in the treatment fish and the most dominant OTU's within this phylum belonged to the γ subclass, and in particular *Acinetobacter*, *Escherichia/Shigella*, *Enterobacter*, *Pseudomonas* and *Pantoea*. The α and β subclasses were also represented and the dominant OTU's recorded from these classes were *Ahrensia* and *Sphingomonas* and *Delftia* and *Pelomonas* respectively. The Spirochaetes were principally represented by the genus *Brevinema*, however *Sphaerochaeta* was also detected. This microbial class was most abundant in the treatment fish, with an overall mean sequence abundance of 3.1%, versus 0.7% in the control fish. Members of the class Bacteroidetes were infrequently recorded, and the dominant OTU's assigned to this class recorded in this study were *Flavobacterium* and *Cloacibacterium*. Similarly, OTU's assigned to the Fusobacteria were poorly represented within all libraries analysed, with *Fusobacterium* and *Cetobacterium* the principal genera detected in the sequence analysis. One of the most dominant OTU's observed in both control and treatment libraries was assigned to Candidate

Division WPS-1, an unclassified phylum, indicating that a large portion of the trout microbiome is still yet to be fully characterized.

a)

Legend:
- Tenericutes (90.8% ± 10.09)
- Firmicutes (7.2% ± 7.82)
- Proteobacteria (0.9% ± 1.33)
- Spirochaetae (0.7% ± 1.00)
- Bacteroidetes (0.02% ± 0.16)
- Actinobacteria (0.01% ± 0.008)
- Deinococcus-Thermus (0.004% ± 0.006)
- Candidate Division WPS-1 (0.12% ± 0.09)
- Fusobacteria (0.001% ± 0.00)

b)

Legend:
- Tenericutes (86.7% ± 6.26)
- Firmicutes (8.2% ± 8.75)
- Proteobacteria (1.5% ± 1.92)
- Spirochaetae (3.1% ± 3.06)
- Bacteroidetes (0.03% ± 0.008)
- Actinobacteria (0.03% ± 0.01)
- Deinococcus-Thermus (0.003% ± 0.002)
- Candidate Division WPS-1 (0.4% ± 0.19)
- Fusobacteria (0.003% ± 0.003)

Figure 3.4. Mean relative % sequence abundance of microbial phyla recorded in distal intestine of rainbow trout fed **a)** control and **b)** treatment diet.

a)
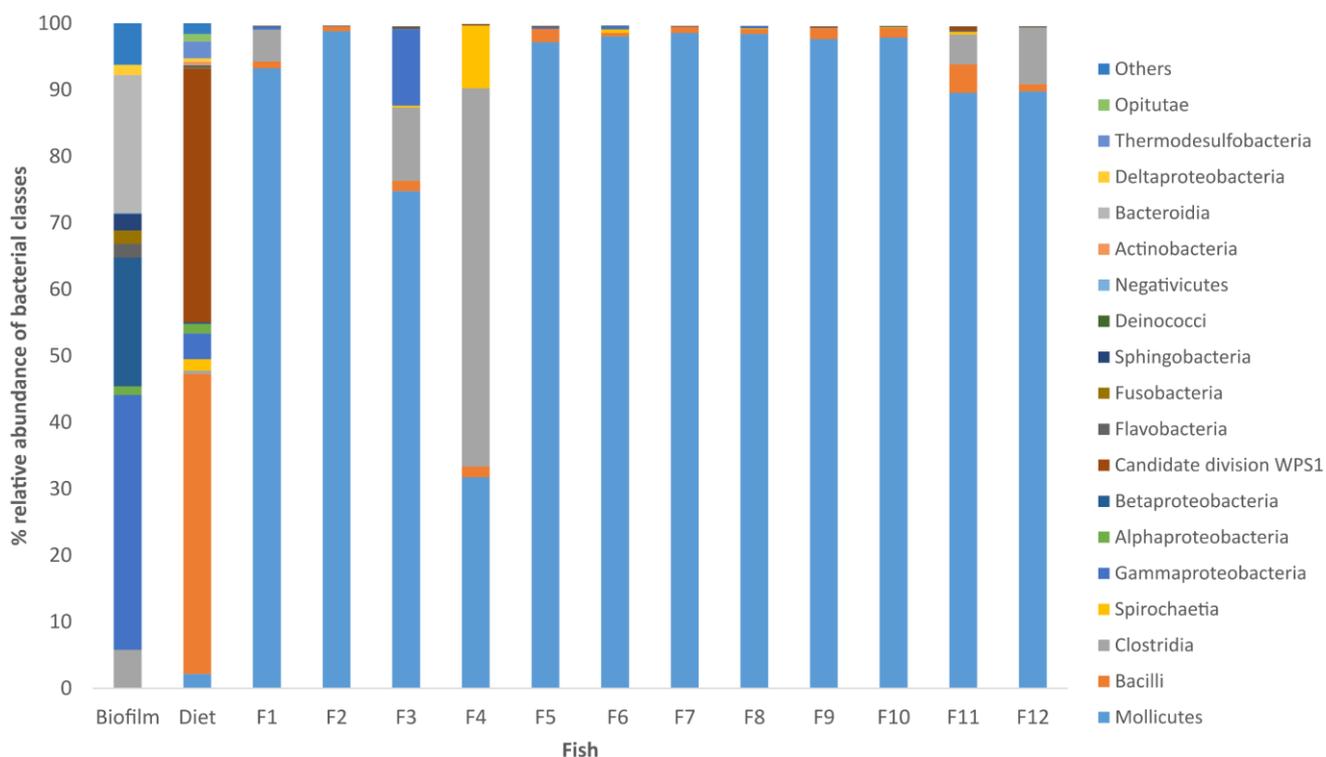
b)
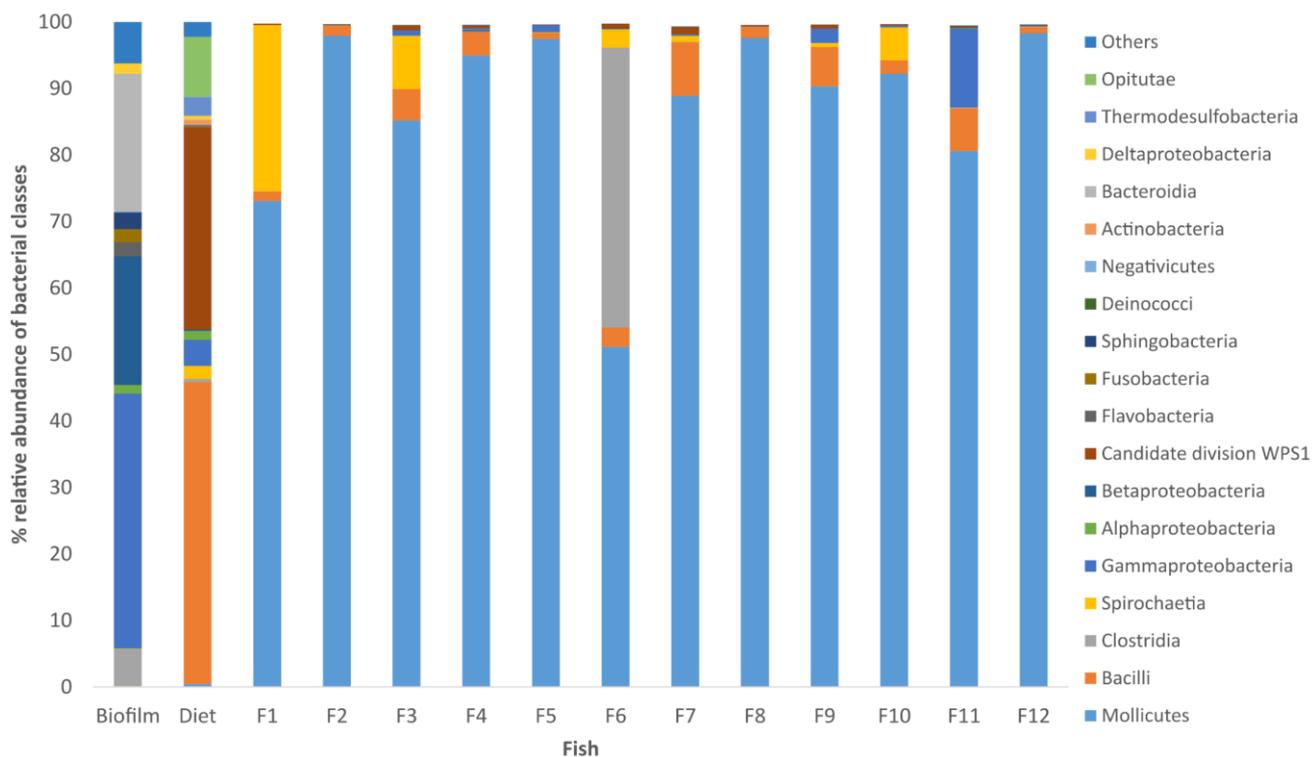
Figure 3.5. Relative % sequence abundance of tank biofilm, diet and intestinal microbial classes in rainbow trout fed **a)** control and **b)** treatment diet.

The PCoA, when visualized based on the ThetaYC distance matrix comparing similarities in community structure, showed that samples were broadly indistinguishable according to diet, with the treatment and control samples clustering close together (Figure 3.6, 3.9 S1). This trend was examined using both the parsimony (Schloss & Handelsman 2006) and unweighted UniFrac (Lozupone & Knight 2005) analyses performed in mothur, and confirmed that the structure of the intestinal microbiome was not significantly different between dietary treatments (ParsSig = 0.269, UWSig = 0.49). The community structure between the feed pellets and the intestinal microbiome was however significantly different when analysed statistically (ParsSig = 0.025, WSig = <0.001, UWSig = 0.004). The microbial community structure of the tank biofilm sample was also found to be significantly different from that of the trout intestinal microbiome samples (WSig = <0.001).
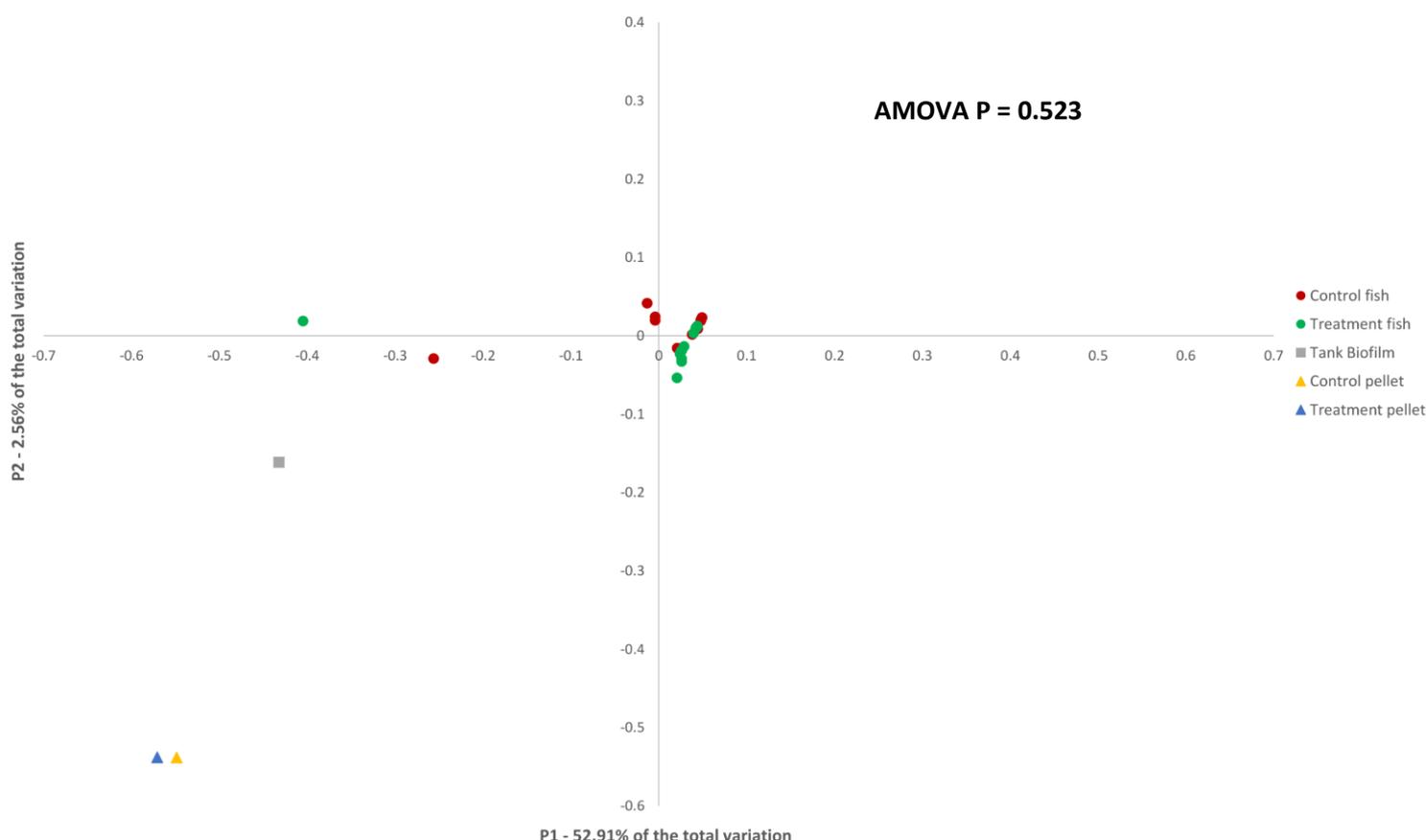
Figure 3.6. Principal coordinate analysis (PCoA) depicting differences in the structure of microbial communities within the distal intestine of control and treatment fish, tank biofilm and feed pellet samples from both diets, based on ThetaYC distance matrix. Each dot represents an individual sample.

Although overall intestinal community structures were not statistically different between control and treatment fish, metastats (White et al 2009) analysis revealed that a number of OTU's were discriminatory according to dietary treatment and hence were differentially represented according to dietary regime (Table 3.3). These OTU's were *Leuconostoc* (p = 0.009), *Streptococcus* (p = 0.009), *Weissella* (p = 0.048), Candidate Division WPS-1 (p = 0.006), *Lactobacillus* (p = 0.010), *Enterobacter* (p = 0.034), *Lactococcus* (p = 0.046) and *Bacillus* (p = 0.047). Furthermore, sequences representing each of these OTU's were significantly more abundant in the intestines of fish in the treatment group (Figure 3.7). Both the LEfSe (Segata et al 2011) and Indicator (McCune et al 2002) statistical algorithms also confirmed the same phylotypes as discriminatory according to diet, with the exception of *Weissella,* where p > 0.05 for both metrics. *Acetanaerobacterium* and *Brevinema* were also selected due to obvious differences in overall mean sequence abundances and because of their high prevalence in the sequence libraries, but these phylotypes were not found to be discriminatory according to diet (Figure 3.8).

Table 3.3. Phylotypes identified as discriminatory according to diet regime by three separate statistical algorithms within mothur (Metastats, LEfSe and Indicator)

Phylotype	p value			
	Metastats	LEfSe	Indicator	Discriminator
Acetanaerobacterium	0.94	-	0.68	-
Brevinema	0.41	-	0.39	-
Streptococcus	0.009	0.009	0.042	Treatment
Leuconostoc	0.009	0.013	0.046	Treatment
Weissella	0.043	-	0.12	Treatment
Candidate division WPS-1	0.006	0.005	0.024	Treatment
Lactobacillus	0.010	0.007	0.034	Treatment
Lactococcus	0.046	0.026	0.058	Treatment
Enterobacter	0.034	0.049	0.078	Treatment

Statistical significance was accepted on two levels; p<0.05 and p<0.01. *Acetanaerobacterium* and *Brevinema* were not discriminatory by diet.
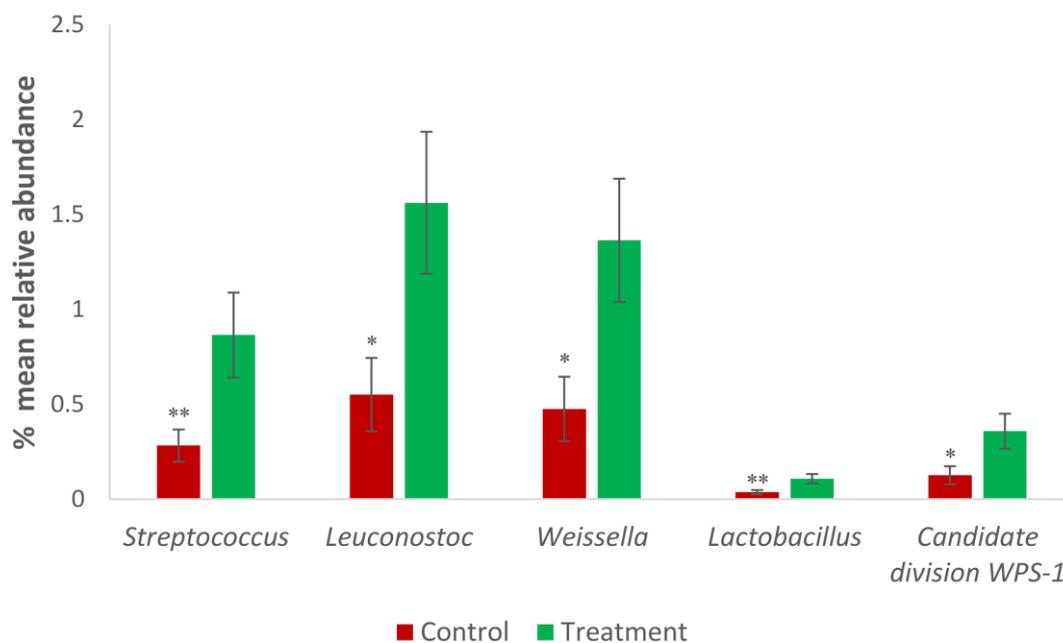
Figure 3.7. Bacterial taxa identified by metastats, LEfSe and Indicator analysis as discriminatory between the intestinal microbiomes of control and treatment fish. The data are plotted as mean percentage relative abundance ± standard error of the mean (SEM). *P<0.05 **P<0.01.
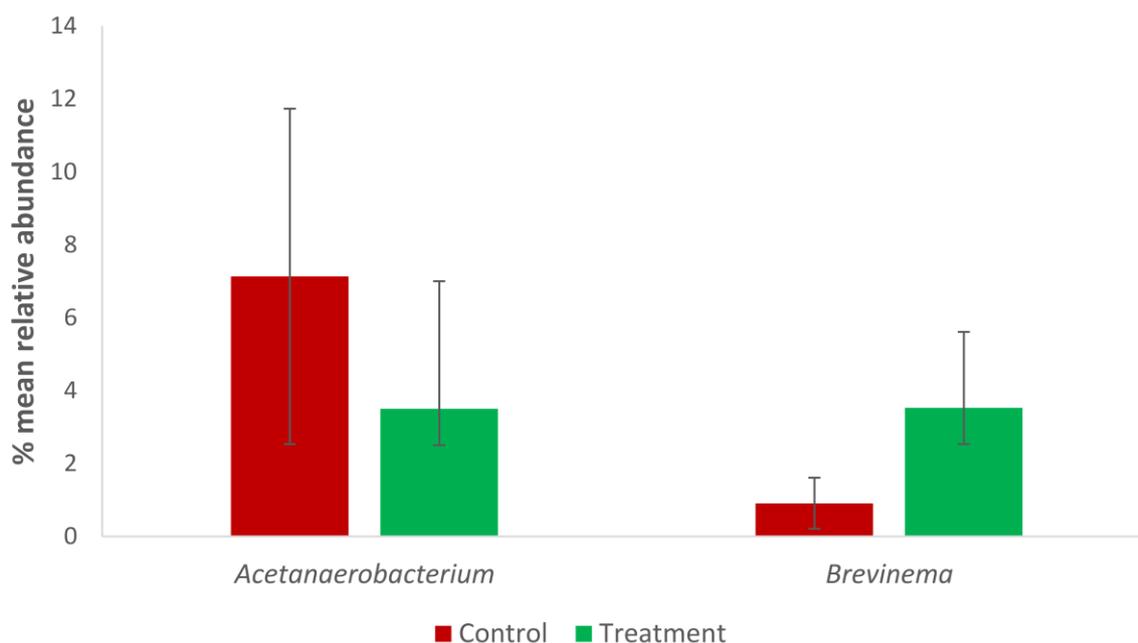
Figure 3.8. Mean relative abundance ± SEM of sequences assigned to *Acetanaeobacterium* and *Brevinema* in intestinal microbiome of rainbow trout fed both control and treatment diets. Taxa not significantly different between groups.

3.5. Discussion

The findings from this study suggest that 5% dietary microalgae supplementation altered levels of bacterial diversity and individual populations of microbes, but not the overall microbial community structure within the intestine of rainbow trout. No significant differences were recorded in growth or condition between control and treatment fish. These results improve our understanding of the structure and diversity of the rainbow trout distal intestinal microbiome and the impact of dietary modification on its composition. Novel dietary supplements and functional feed ingredients will undoubtedly continue to be included in future aquaculture feed formulations as the industry's finite supply of existing sources of fishmeal and fish oil decline. This research found that all of the individual rainbow trout analysed from both test groups possessed broadly similar intestinal microbial community compositions, after fifteen weeks of feeding. However, there were statistically significant differences in the representation of specific bacterial taxa between the control and treatment groups. Within the treatment group a trend towards an increase in microbial diversity was observed, however this pattern was not observed in all fish and consequently was not statistically significant. Nonetheless, the pattern of increased microbial diversity could be indicative of the microbial community within the intestine of these fish responding to the availability of a different dietary ingredient, and perhaps an additional fermentable substrate in the form of the whole cell microalgae supplement.

It has previously been reported that gut microbial diversity may increase from carnivorous to omnivorous to herbivorous fish species (Givens et al 2014, Larsen et al 2014), a pattern similar to that observed in mammals (Ley et al 2008). The reason for this pattern is still poorly understood, but may be correlated with the length of the GI tract in each fish species and hence the overall transit time of food through the gut. In carnivorous fish with short digestive systems, such as rainbow trout, food travels quickly through the gut (Buddington et al 1997) and hence less time may be available for microbial fermentation of dietary ingredients. However, in omnivorous and herbivorous fish, there is a much longer transit time of food through the convoluted GI tract, possibly enabling a greater level of microbial fermentation to occur and precipitating an increase in microbial diversity. Smriga et al (2010) reported that the intestinal microbiome of the herbivorous whitecheek surgeonfish *Acanthurus nigricans*, whose primary diet consists of algae and detritus, exhibited a far greater level of microbial diversity than that of the strictly carnivorous red snapper *Lutjanus bohar*. Similarly, the omnivorous yellowbelly rockcod *Notothenia coriiceps* was shown to possess a greater intestinal microbial diversity than

the carnivorous blackfin icefish *Chaenocephalus aceratus* (Ward et al 2009). In this study, it is not unreasonable to posit that the changes in microbial diversity observed in the treatment group were indicative of the microbiome adapting to metabolizing the whole cell microalgae and its constituent polysaccharides. Whilst gut transit time was not measured, similar trends towards an increased microbial diversity in the intestine of trout fed plant-based diets have been recorded (Desai et al 2012, Green et al 2013). A high level of microbial diversity in the intestine has been advocated as being beneficial to host health in that it provides both a wide range of potential responses to homeostatic perturbations, and can improve the digestion of a greater variety of different dietary ingredients (Backhed et al 2005). The presence of a more diverse microbiome in the microalgae fed fish could therefore represent a reflection of the need for an additional plasticity in the structure of the microbiome, in order to aid the digestion and assimilation of the microalgal meal included in their diet.

The Tenericutes were the dominant microbial phylum in the vast majority of samples, followed by the Firmicutes and Spirochaetes. Within the Tenericutes, the Mollicutes were the most prominent class, with *Mycoplasma* being the dominant genus. This microbe has previously been recorded in the intestinal tract of both marine and freshwater fish species (Holben et al 2002, Moran et al 2005, Kim et al 2007, Bano et al 2007, Suhanova et al 2011, Xing et al 2013, Carda-Dieguez et al 2014, Dzyuba et al 2014). More recent analyses employing high throughput sequencing have reported similar findings to those of the present study, in that the Mycoplasmataceae appear to dominate read libraries from the distal intestinal microbiome of Atlantic salmon (Green et al 2013, Zarkasi et al 2014) and rainbow trout (Lowrey et al 2015, Ozorio et al 2015). *Mycoplasma* do not, however, appear to be significantly affected by diet composition, as they were present in all fish sampled in this trial, irrespective of treatment. Furthermore, large numbers of Tenericutes have been documented in the gut of other aquatic animals such as oysters (King et al 2012) and in terrestrial animals such as pigs (Leser et al 2002).

The genus *Mycoplasma* are nutritionally fastidious Gram-positive bacteria that are closely related to the Bacilli/Clostridium branch of the phylum Firmicutes. They lack cell walls, have a fermentative metabolism, a high G-C content and possess a genome size (~580Kbp) that is amongst the smallest ever to be observed in self-replicating microorganisms. Owing to this extremely small genome, it is unlikely that they perform many complex metabolic functions within the fish intestine, and may primarily be obligate commensal organisms of the gut ecosystem. However, *Mycoplasma* have previously been reported to produce lactic acid and

acetic acid as their major metabolites (Freundt & Razin 1958). It is thus also possible that the dominance of *Mycoplasma* in the intestine of trout is a result of a long established symbiosis in which this microbe benefits from an easy access to a multitude of fermentable substrates (e.g. cytoplasmic secretions) and the fish benefits from the acetic acid and lactic acid metabolites produced as a result. Extreme genome reduction in bacterial symbionts residing within terrestrial animal hosts is a well described phenomenon, which may also occur in rainbow trout.

Previous studies that have analysed the effect of dietary alterations on the rainbow trout intestinal microbiome have reported that whilst slight compositional differences are often observed, the 'core' microbial community remains unaffected, and therefore may be resistant to changes in diet (Wong et al 2013, Zarkasi et al 2014). However, the authors of these studies did report subtle effects of the different diets on the relative abundance of select groups of bacterial taxa. Similarly, the PCoA data obtained in the present study provides evidence of a very minor effect of different diets on the structure of the microbial community within the intestine of rainbow trout, with only a limited number of taxonomic groups being significantly affected by dietary alteration. Furthermore, analysis of the microbial communities of the diets themselves showed that they were very similar in structure, but were significantly different from the fish intestinal samples. It thus appears to be unlikely that the observed differences in microbiome composition between control and treatment fish could be due to the microbial structure of the dietary pellets. Others have also reported that switching dietary regimes, including nutritional substitution, can alter microbial diversity, community membership and/or structure to varying degrees (Ringø & Olsen 1999, Ringø et al 2006, 2010, Dimitroglou et al 2011, Askarian et al 2012, Sullam et al 2012, Bolnick et al 2014).

Statistical analyses revealed that *Streptococcus*, *Leuconostoc*, *Weissella*, *Lactobacillus, Candidate Division WPS-1* and *Lactococcus* were significantly discriminatory between diets in this study. Each of these genera, most of which are members of the LAB, were significantly elevated in the microalgae fed fish. LAB are frequently recorded in the intestines of fish, including rainbow trout, albeit at low levels of abundance (Merrifield et al 2014). More recent research on the effect of diet on the rainbow trout intestinal microbiome, using deep sequencing platforms, has found that this group appears to be amongst the most responsive to dietary alterations. Ingerslev et al (2014a, b) reported that *Streptococcus*, *Leuconostoc*, *Weissella* and *Lactobacillus* were receptive to dietary shifts, and were significantly elevated in the microbiome of trout fed high levels of plant-based ingredients. Similarly, both Desai et al

(2012) and Wong et al (2013) reported that *Lactobacillus*, *Streptococcus*, *Weissella*, *Clostridia* and *Staphylococcus* were discriminatory according to plant based and grain based diets respectively. The same microbial groups, with the exception of *Staphylococcus*, were discriminatory by diet in the present study, indicating the possible development of a distinct pattern of dietary influences on LAB populations in the rainbow trout intestine, in spite of their perceived rarity within this ecosystem.

The LAB are generally considered to be beneficial organisms associated with a healthy intestinal epithelium, and many of the genera recorded in this research have been tested elsewhere for their potential probiotic capabilities in rainbow trout aquaculture (Joborn et al 1997, Irianto & Austin 2002, Panigrahi et al 2004, 2005, Kim & Austin 2006, 2008, Balcazar et al 2007, 2009, Vendrell et al 2008, Merrifield et al 2010, Perez-Sanchez et al 2011). LAB are hypothesized to improve the health of rainbow trout in aquaculture by enhancing feed conversion efficiency and conferring protection against pathogenic bacteria via mechanisms of competitive exclusion. In addition, their ability to produce organic acids (e.g. acetic acid, lactic acid) and compounds such as bacteriocins and enzymes can further protect the intestinal epithelium and aid in the digestion of resistant dietary ingredients (Nayak 2010). The LAB have however been observed to represent only a minor constituent of the fish intestinal microbiome and so potential methods of modulating and enriching these populations are of great interest in improving intestinal health and consequently fish performance in aquaculture.

Overall, the results presented showed that the inclusion of whole-cell microalgae in the diet of farmed rainbow trout did not impair their growth or negatively impact the distal intestinal microbiome. The dominance of *Mycoplasma* in the microbial libraries of all fish analysed suggests that this phylotype is well adapted to life in the rainbow trout intestine, and hence further research into its potential functional role is undoubtedly required. The altered microbial diversity observed in the microalgae fed fish is suggestive of a flexibility in the intestinal microbiome of these fish, which may represent a response to the breakdown and digestion of this novel dietary ingredient. Whilst the 'global' microbiome structure was similar in both groups, there were statistically significant differences noted in community membership, with distinct microbial groups observed to be discriminatory according to diet, particularly members of the LAB such as *Weissella*, *Streptococcus*, *Lactococcus*, *Lactobacillus* and *Leuconostoc*. This represents a further indication of a possible, albeit subtle, dietary effect of the microalgae on these populations. The potential manipulation of microbial communities through dietary supplementation may represent a promising method for improving gut health and hence

nutrient utilization in farmed rainbow trout. Whilst the data presented is certainly supportive of the inclusion of microalgae in farmed rainbow trout diets, further work is required to clarify the optimal level of inclusion required to beneficially modulate the intestinal microbiome of these fish.

3.6. Acknowledgements

3.7. Conflicts of interest

The authors declare no conflicts of interest

3.8. References

Askarian, F., Zhou, Z., Olsen, R.E., Sperstad, S. and Ringø, E. (2012) Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and in vitro growth inhibition of four fish pathogens. *Aquaculture* **326,** 1-8

Austin, B. (2006) The bacterial microflora of fish, revised. *The Scientific World Journal,* **6**, 931-945

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. and Gordon, J.I. (2005) Host-bacterial mutualism in the human intestine. *Science* **307,** 1915-1920

Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Girones, O. and Muzquiz, J.L. (2007) Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). *FEMS Immunology and Medical Microbiology* **51**, 185-193

Balcazar, J.L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I. and Muzquiz, J.L. (2009) Effect of *Lactococcus lactis* CLFP 100 and *Leuconostoc mesenteroides* CLFP 196 on *Aeromonas salmonicida* infection in brown trout (*Salmo trutta*). *Journal of Molecular Microbiology and Biotechnology* **17**, 153-157

Bano, N., deRae Smith, A., Bennett, W., Vasquez, L. and Hollibaugh, J.T. (2007) Dominance of Mycoplasma in the guts of the Long-Jawed Mudsucker, *Gillichthys mirabilis*, from five California salt marshes. *Environmental Microbiology* **9**, 2636-2641

Bolnick, D.I., Snowberg, L.K., Hirsch, P.E., Lauber, C.L., Knight, R., Caporaso, J.G. and Svanback, R. (2014) Individuals' diet diversity influences gut microbial diversity in two freshwater fish (threespine stickleback and Eurasian perch). *Ecology Letters* **17**, 979-987

Buddington, R.K., Krogdahl, A. and Bakke-McKellep, A.M. (1997) The intestines of carnivorous fish: structure and functions and the relations with diet. *Acta Physiologica Scandinavica Supplementum* **638**, 67-80

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G. and Knight, R. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal,* **6**, 1621-1624

Carda-Dieguez, M., Mira, A. and Fouz, B. (2014) Pyrosequencing survey of intestinal microbiota diversity in cultured sea bass (*Dicentrarchus labrax*) fed functional diets. *FEMS Microbiology Ecology* **87**, 451-459

Cerezuela, R., Fumanal, M., Tapia-Paniagua, S.T., Meseguer, J., Morinigo, M.A. and Esteban, M.A. (2012) Histological alterations and microbial ecology of the intestine in gilthead sea bream (*Spaurus aurata* L.) fed dietary probiotics and microalgae. *Cell and Tissue Research* **350**, 477-489

Choat, J.H. and Clements, K.D. (1998) Vertebrate herbivores in marine and terrestrial environments: A nutritional ecology perspective. *Annual Review of Ecology and Systematics* **29**, 375-403

Clements, K.D., Pasch, I.B.Y., Moran, D. and Turner, S.J. (2007) Clostridia dominate 16S rRNA libraries prepared from the hindgut of temperate marine herbivorous fishes. *Marine Biology* **150**, 1431-1440

Clements, K.D., Angert, E.R., Montgomery, W.L. and Choat, J.H. (2014) Intestinal microbiota in fishes: what's known and what's not. *Molecular Ecology* **23**, 1891-1898

Desai, A.R., Links, M.G., Collins, S.A., Mansfield, G.S., Drew, M.D., Van Kessel, A.G. and Hill, J.E. (2012) Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **350**, 134-142

Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C.L., Guroy, D. and Davies, S.J. (2011) Microbial manipulations to improve fish health and production: A Mediterranean perspective. *Fish and Shellfish Immunology* **30**, 1-16

Dzyuba, E.V., Suhanova, E.V., Denikina, N.N. and Belkova, N.L. (2014) Comparative analysis of gut microbiocenoses of salmonid fish with different feeding strategies. *Doklady Biological Sciences: Proceedings of the Academy of Sciences of the USSR, Biological Sciences Sections / Translated from Russian* **11**, 2429-2433

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. and Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194-200

Freundt, E.A. and Razin, S. (1958) Genus *Mycoplasma.* In: *Bergey's Manual of Systematic Bacteriology*, vol. 1. (Krieg, N.R. and Holt, J.G. Eds.) pp. 742-770, Williams and Wilkins, Baltimore, USA

Gatesoupe, F.J., Huelvan, C., Le Bayon, N., Sevre, A., Aaasen, I.M., Degnes, K.F., Mazurais, D., Panserat, S., Zambonino-Infante, J.L. and Kaushik, S.J. (2014) The effects of dietary carbohydrate sources and forms on metabolic response and intestinal microbiota in sea bass juveniles, *Dicentrarchus labrax. Aquaculture* **422**, 47-53

Ghanbari, M., Kneifel, W. and Domig, K. (2015) A new view of the fish gut microbiome: Advances from next-generation sequencing. *Aquaculture* **448**, 464-475

Givens, C.E., Ransom, B., Bano, N. and Hollibaugh, J.T. (2014) A fish tale: comparison of the gut microbiomes of 12 finfish and 3 shark species. *Journal of Marine Ecology Progression Series* **518**, 209-223

Green, T.J., Smullen, R. and Barnes, A.C. (2013) Dietary soybean protein concentrate-induced intestinal disorder in marine farmed Atlantic salmon, *Salmo salar* is associated with alterations in gut microbiota. *Veterinary Microbiology* **166**, 286-292

Holben, W.E., Williams, P., Gilbert, M., Saarinen, M., Sarkilahti, L.K. and Apajalahti, J.H., (2002) Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. *Microbial Ecology* **44**, 175-185

Ingerslev, H., von Gersdorff Jørgensen, L., Lenz Strube, M., Larsen, N., Dalsgaard, I., Boye, M. and Madsen, L. (2014a) The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. *Aquaculture,* **424**, 24-34

Ingerslev, H.C., Strube, M.L., Jørgensen, L., Dalsgaard, I., Boye, M. and Madsen, L. (2014b) Diet type dictates the gut microbiota and the immune response against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology* **40**, 624-633

Irianto, A. and Austin, B. (2002) Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **25**, 333-342

Joborn, A., Olssen J.C., Westerdahl, A., Conway, P.L. and Kjelleberg, S. (1997) Colonization in the fish intestinal tract and production of inhibitory substances in intestinal mucous and faecal extracts by *Carnobacterium* sp. Strain K1. *Journal of Fish Diseases* **20**, 383-392

Kim, D.H. and Austin B. (2006) Innate immune responses in rainbow trout (*Oncorhynchus mykiss*, Walbaum) induced by probiotics. *Fish and Shellfish Immunology* **21**, 513-524

Kim, D.H., Brunt, J. and Austin, B. (2007) Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). *Journal of Applied Microbiology* **102**, 1654-1664

Kim., D.H. and Austin, B. (2008) Characterization of probiotic carnobacteria isolated from rainbow trout (*Oncorhynchus mykiss*) intestine. *Letters in Applied Microbiology* **47**, 141-147

King, G.M., Judd, C., Kuske, C.R. and Smith, C. (2012) Analysis of stomach and gut microbiomes of the eastern oyster (*Crossotrea virginica*) from coastal Louisiana, USA. *PLOS ONE* **7:** e51475

Kormas, K.A., Meziti, A., Mente, E. and Frentzos, A. (2014) Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*). *Microbiology Open* **3**, 718-728

Larsen, A.M., Mohammed, H.H. and Arias, C.R. (2014) Characterization of the gut microbiota of three commercially valuable warm water species. *Journal of Applied Microbiology* **116**, 1396-1404

Leser, T.D., Amenuvor, J.Z., Jensen, T.K., Lindecrona, R.H., Boye, M. and Moller, K. (2002) Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. *Applied and Environmental Microbiology* **68**, 673-690

Ley, R.E., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R. and Bircher, S. (2008) Evolution of mammals and their gut microbes. *Science* **320**, 1647-1651

Lowrey, L., Woodhams, D.C., Tacchi, L. and Salinas, I. (2015) Topographical mapping of the rainbow trout microbiome reveals a diverse bacterial community in the skin with antifungal properties. *Applied and Environmental Microbiology* **81**, 6915-6925

Lozupone, C. and Knight, R. (2005) Unifrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* **71**, 8228-8235

Mansfield, G.S., Desai, A.R., Nilson, S.A., Van Kessel, A.G., Drew, M.D. and Hill, J.E. (2010) Characterization of rainbow trout (*Oncorhynchus mykiss*) intestinal microbiota and inflammatory marker gene expression in a recirculating aquaculture system. *Aquaculture* **307**, 95-104

McCune, B., Grace, J.B. and Urban, D.L. (2002) Analysis of ecological communities. MjM Software, Gleneden Beach, Oregon USA

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bogwald, J., Castex, M. and Ringø, E. (2010) The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* **302**, 1-18

Merrifield, D.L., Olsen, R.E., Myklebust, R. and Ringø, E. (2011) Dietary effects of soybean products on gut histology and microbiota of fish. In: *Soybean and Nutrition* (El-Shemy, H. Ed.), pp. 231-251, InTech, Croatia

Merrifield, D.L., Balcazar, J.L., Daniels, C., Zhou, Z., Carnevali, O., Sun, Y.Z., Hoseinifar, H. and Ringø, E. (2014) Indigenous lactic acid bacteria in fish and crustaceans. In: *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. (1st edn.) (Merrifield, D. and Ringø, E. Eds.) pp. 128-168, WILEY Blackwell, Oxford, UK

Miyake, S., Ngugi, D.K. and Stingl, U. (2015) Diet strongly influences the gut microbiota of surgeonfishes. *Molecular Ecology* **24**, 656-672

Moran, D., Turner, S.J. and Clements, K.D. (2005). Ontogenetic development of the gastrointestinal microbiota in the marine herbivorous fish *Kyphosus sydneyanus*. *Microbial Ecology* **49**, 590-597

National Research Council (2011) *Nutrient Requirements of Fish and Shrimp*. National Academy Press, Washington D.C.

Nayak, S.K. (2010) Role of gastrointestinal microbiota in fish. *Aquaculture Research* **41**, 1553-1573.

Ozorio, R.O.A., Kopecka-Pilarczyk, J., Peixoto, M.J., Lochmann, R., Santos, R.J., Santos, G., Weber, B., Calheiros, J., Ferrez-Arruda, L., Vaz-Pires, P. and Goncalves, J.F.M. (2015) Dietary probiotic supplementation in juvenile rainbow trout (*Oncorhynchus mykiss*) reared under cage culture production: effects on growth, fish welfare, flesh quality and intestinal microbiota. *Aquaculture Research* **doi:** 10.1111/are.12724

Panigrahi, A., Kiron, V., Kobayashi, T., Puangkaew, J., Satoh, S. and Sugita, H. (2004) Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. *Veterinary Immunology and Immunopathology* **102**, 379-388

Panigrahi, A., Kiron, V., Puangkaew, J., Kobayashi, T., Satoh S. and Sugita, H. (2005) The viability of probiotic bacteria as a factor influencing the immune response in rainbow trout *Oncorhynchus mykiss*. *Aquaculture* **243**, 241-254

Perez-Sanchez, T., Balcazar, J.L., Merrifield, D.L., Carnevali, O., Gioacchini, G., de Blas, I. and Ruiz-Zarzuela, I. (2011) Expression of immune related genes in rainbow trout (*Oncorhynchus mykiss*) induced by probiotic bacteria during *Lactococcus garviae* infection. *Fish and Shellfish Immunology* **31**, 196-201

Rambaut, A. (2009) FigTree v.1.4.2. Available online: http://tree.bio.ed.ac.uk/software/figtree

Ringø, E. and Olsen, R.E. (1999) The effect of diet on aerobic bacterial flora associated with intestine of Arctic charr (*Salvelinus alpinus* L.). *Journal of Applied Microbiology* **86**, 22-28

Ringø, E., Sperstad, S., Myklebust, R., Refstie, S. and Krogdahl, A. (2006) Characterization of the microbiota associated with the intestine of Atlantic cod (*Gadhus morhua* L.): The effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture* **261**, 829-841

Ringø, E., Olsen, R.E., Gifstad, T.O., Dalmo, R.A., Amlund, H., Hemre, G.I. and Bakke, A.M. (2010) Prebiotics in aquaculture: a review. *Aquaculture Nutrition* **16**, 117-136

Romero, J., Ringø, E. and Merrifield, D. (2014) The gut microbiota of fish. In: *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. (1st edn.) (Merrifield, D. and Ringø, E. Eds.) pp. 75-100, WILEY Blackwell, Oxford, UK

Schloss, P.D. and Handelsman, J. (2006) Introducing TreeClimber, a test to compare microbial community structures. *Applied and Environmental Microbiology* **72**, 2379-2384

Schloss, P.D. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**, 7537-7541

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S. and Huttenhower, C. (2011) Metagenomic biomarker discovery and explanation. *Genome Biology* **12**: R60, **doi**: 10.1186/gb-2011-12-6-r60

Smriga, S., Sandin, S. and Azam, F. (2010) Abundance, diversity and activity of microbial assemblages associated with coral reef fish guts and feces. *FEMS Microbiology Ecology* **73**, 31-42

Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T., Appel, K.F. and Gram, L. (2000) The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. *Aquaculture* **182**, 1-15

Suhanova, E.V., Dzyuba, E.V., Triboy, T.I., Nikiforova, T.I., Denikina, N.N. and Belkova, N.L. (2011) Molecular genetic and culture diagnosis of Mycoplasma in fish family Thymallidae. *Doklady Biological Sciences: Proceedings of the Academy of Sciences of the USSR, Biological Sciences Sections / Translated from Russian* **440**, 287-289

Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'Connor, M.P., Rosen, G.L., Knight, R., Kilham, S.S. and Russell, J.A. (2012) Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Molecular Ecology* **21**, 3363-3378

Tacon, A. G. J. and Metian, M. (2008) Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture* **285**, 146-158

Vendrell, D., Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Girones, O. and Muzquiz, J.L. (2008) Protection of rainbow trout (*Oncorhynchus mykiss*) from lactococcosis by probiotic bacteria. *Comparative Immunology, Microbiology and Infectious Diseases* **31**, 337-345

Ward, N., Steven, B., Penn, K., Methe, B. and Detrich, W. (2009) Characterization of the intestinal microbiota of two Antarctic notothenioid fish species. *Extremophiles* **13**, 679-685

Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**, 697-703

White, J.R., Nagarajan, N. and Pop, M. (2009) Statistical methods for detecting differentially abundant features in clinical metagenomics samples. *PLOS Computational Biology* **5**, e1000352

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T., Wiens, G.D., Snekvik, K., Rawls, J.F. and Good, C. (2013) Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. *Applied and Environmental Microbiology* **79**, 4974-4984

Xing, M., Hou, Z., Yuan, J., Liu, Y., M Qu, Y. and Liu, B. (2013) Taxonomic and functional metagenomics profiling of gastrointestinal tract microbiome of the farmed adult turbot (*Scopthalmus maximus*) *FEMS Microbiology Ecology* **86**, 432-443

Yue, J.C. and Clayton, M.K. (2005) A similarity measure based on species proportions. *Communications in Statistics Theory and Methods* **34**, 2123-2131

Zarkasi, K., Abell, G., Taylor, R., Neuman, C., Hatje, E., Tamplin, M., Katouli, M. and Bowman, J.P. (2014) Pyrosequencing based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system. *Journal of Applied Microbiology* **117**, 18-27

3.9. Supplementary information
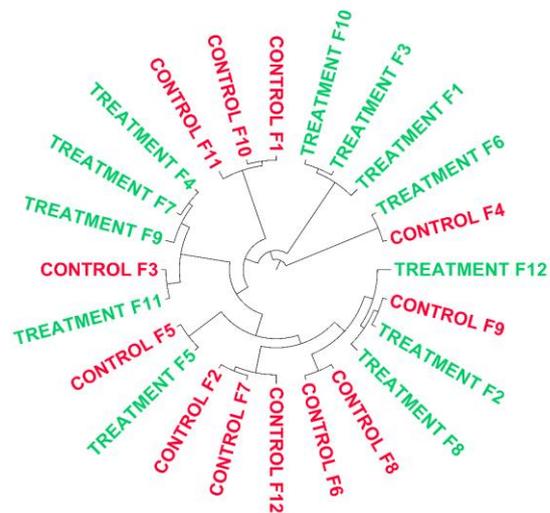
Figure 3.9. (S1) Clustering of samples describing the dissimilarity in the structure of the intestinal microbiome of control and treatment groups according to ThetaYC distances.

a)
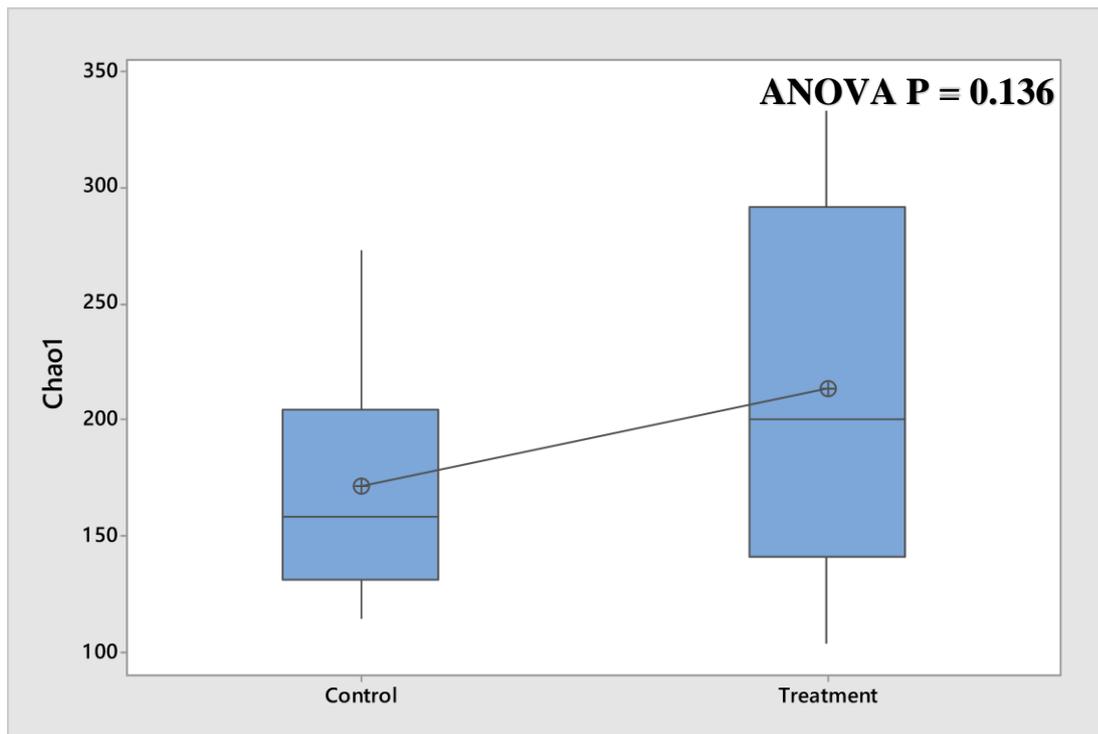
b)
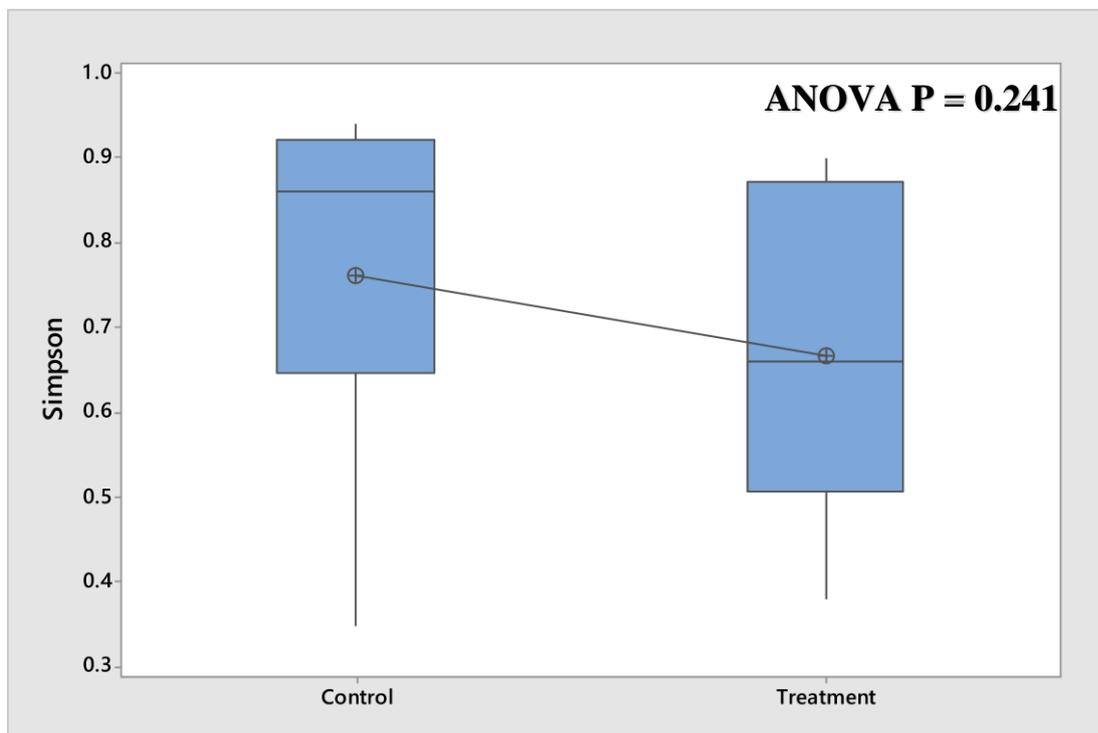
Figure 3.10 (S2) Box plots (median, quartiles and range values) of **a)** Chao1 richness and **b)** Simpson's diversity index of intestinal microbiomes of rainbow trout fed with control and treatment diets.

Chapter 4

Phylogenetic and functional characterization of the distal intestinal microbiome of rainbow trout from both farm and aquarium settings

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4.1. Abstract

The intestine of rainbow trout is known to host complex microbial communities that are hypothesized to influence their development, digestion, nutrition and immunity. In order to better understand and exploit these communities and their impact on fish health, it is vital to determine their structure, diversity and potential functional capacity. This study focused on comparing the phylogenetic composition and functional potential of the intestinal microbiome of rainbow trout sourced from both farm and aquarium settings. Samples of distal intestinal contents were collected from fish from both environments and subjected to high throughput 16S rRNA Illumina sequencing, to accurately determine the composition of the intestinal microbiome. The predominant phyla identified from both groups were Tenericutes, Firmicutes, Proteobacteria, Spirochaetae and Bacteroidetes. A novel metagenomic tool, PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states), was used to determine the functional potential of the bacterial communities present in the rainbow trout intestine. Functional metagenomic pathways concerning membrane transport activity were principally represented in the intestinal microbiome of all fish samples. Furthermore, this analysis revealed that gene pathways relating to metabolism, and in particular amino acid and carbohydrate metabolism, were upregulated in the rainbow trout intestinal microbiome. The results suggest that the structure of the intestinal microbiome in farmed rainbow trout may be broadly similar regardless of where the fish are located. Differences were however noted in the microbial community membership within the intestine of farm and aquarium based fish, suggesting that more sporadic taxa could be unique to each environment and may have the ability to colonize the rainbow trout GI tract. Finally, the functional analysis provides evidence that the microbiome of farmed rainbow contains genes that could contribute to the metabolism of dietary ingredients and therefore may actively influence the digestive process in these fish.

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4.2. Introduction

It is now well documented that animals harbour a vast number of microorganisms, collectively termed the microbiome, both on their body surfaces and particularly within their GI tract. The most numerous of these microorganisms are the Bacteria; however, yeasts, viruses, archaea and protozoans also inhabit these ecosystems. As molecular technologies develop and become more advanced, we are beginning to unravel the true diversity of these communities and the potential impact that they may have on host development, nutrition, disease resistance and immunity. Despite the widespread adoption of these technologies to study the microbiome of terrestrial animals, comparatively little is known of the intestinal microbiome of fish, and in particular economically important farmed fish species such as rainbow trout *Oncorhynchus mykiss*.

Rainbow trout are reared in a number of different aquaculture settings, such as earthen ponds, raceways, inshore tank systems and in freshwater/seawater cages. It has been hypothesized that these different farming environments can shape the composition of the gastrointestinal microbiome, with different taxa dominating according to the geographical location and environmental conditions of the farm in question (Cahill 1990, Spanggaard et al 2000, Nayak 2010). The 'core' microbiome concept proposes that individual hosts maintained under the same husbandry conditions, in the same environment and location will share similar microbial taxonomic compositions (Turnbaugh & Gordon 2009, Wong et al 2013). Novel nutritional strategies such as pro-, pre- and synbiotic feeds have been developed which aim to modulate the gut microbiota, especially in light of the industry's commitment to reduce its use of both antibiotics, and fish meal/oil sourced from wild pelagic fisheries. Therefore, it is important to determine the potential existence of a core microbiome amongst rainbow trout, and whether such a core is shared even amongst fish reared in different geographical locations and farming environments. This information could aid in refining nutritional strategies that aim to harness the potential of these communities, by improving our presently limited understanding of the normal or baseline composition of the rainbow trout intestinal microbiome.

More recent studies that have used high throughput sequencing technologies have shown that the fish intestine harbours a more complex and diverse microbiome than previously considered (Llewellyn et al 2014, 2015, Lowrey et al 2015, Ghanbari et al 2015). Some studies have shown that bacterial populations within teleost fish intestines can be altered in response to different dietary ingredients (Desai et al 2012, Carda-Dieguez et al 2014, Kormas et al 2014, Miyake et al 2015). Others have demonstrated that the core microbiome is resistant to changes in diet and rearing density, and that community profiles of individual fish, reared in the same aquaculture setting, can attain remarkable levels of uniformity (Wong et al 2013, Zarkasi et al 2014). However, it remains unclear whether the structure of the microbiome varies between individual fish of the same species reared in different farming environments, or whether these fish harbour a specialized microbiota independent of geographical location.

Furthermore, although a clearer picture has emerged of the extent of the microbial diversity within the rainbow trout intestinal microbiome, thus far no reports have been documented concerning the functional capability of these communities. Therefore, this study employed a novel but well validated computational approach, PICRUSt (Langille et al 2013) (http://picrust.github.io/picrust), to predict the potential functional capacity of the intestinal microbiome and to complement the phylogenetic data generated. PICRUSt uses an extended ancestral-state reconstruction algorithm to predict which gene families are present within 16S rRNA libraries, and then combines those gene families to estimate the composite metagenome. This approach has been used successfully to study the cecal microbiome of the farmed broiler chicken *Gallus gallus domesticus* (Corrigan et al 2015, Pourabedin & Zhao 2015, Shaufi et al 2015). A detailed knowledge of the phylogenetic profile and functional capacities of the intestinal microbiota is extremely important in order to aid our understanding of the role of these microorganisms in fish health and digestive physiology.

The aim of the present study was therefore to produce an in-depth taxonomic and functional characterization of the rainbow trout intestinal microbiome from individual fish maintained in separate rearing environments. It was hypothesized that the gut bacterial communities would differ between the two farming locations due to the inherent differences in each system's environment. This research therefore would test whether the diversity and structure of these communities was affected by differences in rearing environment, in addition to elucidating fundamental information about their potential functional role within the intestinal ecosystem.

4.3. Materials and methods

4.3.1. Sample collection

A total of twelve rainbow trout were collected from a freshwater fish farm based on Loch Awe, Argyll, Scotland (Figure 4.1). Six fish were each randomly sampled from two separate pens, identified as A and B. The water temperature at the time of sampling was 9.4°C. The fish from each pen originated from different egg sources, but were raised at the same hatchery. All individuals collected on the day of sampling were apparently healthy, that is, with no visual signs of disease or parasites on the skin or internal organs. All fish were fed the same commercial pelleted feed (Skretting Royale Horizon HS™). The mean weight (± SD) of the fish from pen's A and B was 119 ± 24 g and 79 ± 10 g respectively at the time of sampling (Table 4.1).

Figure 4.1. Layout of Braevallich rainbow trout farm at Loch Awe, Dalmally, UK on day of sampling.

A further nine fish were collected from the Aquatic Research Facility (ARF) at the Institute of Aquaculture, University of Stirling, Scotland, UK. Three fish were sampled from each of three separate tanks of 100 L capacity. These tanks were maintained on a flow through system, with an ambient water temperature (11.8°C), and a photoperiod of 12 h light 12 h dark. All of these fish originated from a local trout farm in Perthshire, UK. All were fed the same conventional pelleted feed (Aquate® Alltech Biotechnology Inc., Nicholasville, KY, USA). The mean weight (± SD) of these fish was 191 ± 45 g at the time of sampling (Table 4.1). In addition, two samples

of the pelleted feed and a single tank biofilm sample were taken to compare against the microbiome of the rainbow trout intestine. The tank biofilm and diet samples analysed were the same as those examined in chapter 3.

All fish were sacrificed with a lethal dose of the anaesthetic benzocaine (Sigma Aldrich®, Poole, UK) and swabbed with 100% ethanol before dissection of the ventral surface. The tissues surrounding the visceral fat were aseptically removed and the distal intestine identified. The distal gut contents from a tissue section of approximately 2.5 cm in length (~150 mg) were removed by gently massaging the tissue with a sterile forceps and were placed into sterile 2 ml capped microtubes (Alpha laboratories®, Eastleigh, UK) containing 1 ml of buffer ASL (Qiagen, Hilden, Germany). The tissue was then incised and washed with a sterile 0.85% (w/v) salt solution, and the intestinal mucous was carefully removed from the gut wall. This material was placed into the same tube as the gut contents. All tubes were immediately placed on dry ice after sampling, before being transferred to the laboratory for subsequent same-day DNA extraction.

Table 4.1. Morphometric measurements of fish sampled

Sample	Length (cm)	Weight (g)	Egg Source	Origin
DF AF1	22	143	RA	FR
DF AF2	22.2	143	RA	FR
DF AF3	20	116	RA	FR
DF AF4	21.5	129	RA	FR
DF AF5	19	90	RA	FR
DF AF6	17.9	90	RA	FR
Mean Pen A	**20.43**	**118.5**	**-**	**-**
SD Pen A	**1.75**	**24.25**	**-**	**-**
DF BF1	18.5	88	SS	FR
DF BF2	18.2	88	SS	FR
DF BF3	18.5	77	SS	FR
DF BF4	17.5	68	SS	FR
DF BF5	18.2	88	SS	FR
DF BF6	17.5	66	SS	FR
Mean Pen B	**18.05**	**79.1**	**-**	**-**
SD Pen B	**0.45**	**10.36**	**-**	**-**
AQ F1	23.2	166.9	CM	CM
AQ F2	22.3	192.1	CM	CM
AQ F3	19.8	127.6	CM	CM
AQ F4	25.3	230	CM	CM
AQ F5	24	208	CM	CM
AQ F6	25.5	221.1	CM	CM
AQ F7	26.3	265.6	CM	CM
AQ F8	20.1	151.9	CM	CM
AQ F9	23.2	151.5	CM	CM
Mean AQ	**23.3**	**190.5**	**-**	**-**
SD AQ	**2.28**	**44.6**	**-**	**-**

DF = Fish sampled from fish farm. AQ = Fish sampled from aquarium.

RA = Danish hatchery. SS = Seven Springs hatchery

FR = Frandy Farm. CM = College Mill farm

4.3.2. DNA extraction

A total of 150 mg of intestinal content material from each individual fish suspended in 1 ml of buffer ASL (Qiagen), was processed for DNA extraction. The extractions were performed on the same day as sampling to ensure optimal sample integrity. A sample of 1 ml buffer ASL was processed as a negative control. Samples were firstly disrupted using a Mini- Bead-Beater 16 (Biospec Products Inc.) at maximum speed for four separate cycles of 35 s each. Samples were allowed to settle, and total genomic DNA was extracted and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), with the following modifications to the manufacturer's protocol : 150 mg starting material in 1 ml buffer ASL; suspension heated at 95°C for 10 min to improve lysis of Gram positive bacteria; 0.5 Inhibitex tablet per sample in 700 µl supernatant ; final sample elution volume of 50 µl. Intestinal content samples typically contain many compounds that can degrade DNA and inhibit downstream enzymatic reactions. The QIAamp kit is specifically designed to remove these inhibitors and final purified eluates are enriched for microbial community DNA. After extraction, the DNA concentration of all samples was determined both spectrophotometrically (NanoDrop 1000®, Thermo Scientific Ltd., DE, USA) and fluorometrically (Qubit®, Life Technologies Ltd., Paisley, UK) to ensure optimal DNA purity, and samples were stored at -20°C for downstream processing.

4.3.3. 16S rRNA PCR and Illumina sequencing

A PCR was firstly carried out using universal eubacterial primers 27F AGAGTTTGATCMTGGCTAG and 1492R TACGGYTACCTTGTTACGACTT (Weisburg et al 1991) that target the full length bacterial 16S rRNA gene sequence, to confirm the presence of ample microbial community DNA and to rule out the presence of any potential inhibitory compounds. The extraction from the sample containing buffer ASL only was included in this PCR as a negative control. The PCR conditions for this confirmatory reaction were as follows: denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 2 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min, before final elongation of 72°C for 10 min. Products were then visualized on a 1.5% (w/v) agarose gel, run at 100V for approximately 75 min. The presence of a single strong PCR product of ~1500bp was considered to be indicative of the presence of microbial community DNA. Illumina libraries were prepared following the method described by Caporaso et al (2012) using the NEXTflex 16S Amplicon-Seq kit (Bioo Scientific, Austin USA). A total of 12.5 ng of template DNA was used for each individual sample and the V4 hypervariable region of the bacterial 16S rRNA

gene (length 292bp) was amplified using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (GATC Biotech AG, Konstanz, Germany). The PCR conditions were as follows: initial denaturation at 95ºC for 5 min, 25 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 30 s; followed by a final extension step at 72ºC for 5 min. All samples were amplified in triplicate and all products purified using Agencourt AMpure XP beads (Beckman Coulter (UK) Ltd.).

The products of the first PCR served as a template for a second PCR with the same conditions as the first, however the number of cycles was reduced to eight, and Illumina sequencing adapters were added to the primers in the reaction mix. Following amplification, PCR products were purified using Agencourt AMpure XP (Beckman Coulter (UK) Ltd.) with a modified 1:1 volume of PCR product to AMpure XP beads. Purified amplicons were quantified with Qubit®, pooled in equal concentration and the final quality of the pooled library was validated using an Agilent Bioanalyzer 2100® (Agilent Technologies, Waldbronn, Germany). The final library was prepared and sequenced by GATC Biotech AG (Konstanz, Germany) using the Illumina MiSeq® NGS system.

4.3.4. Bioinformatics

Demultiplexing was performed with Casava v. 1.8, and reads representing the PhiX or reads not matching indices were removed. FastQC (Andrews 2010) was used to assess the overall quality of all sample libraries, and a threshold Phred score ($Q \geq 25$) was set. The open-source software, mothur (Schloss 2009), was used to process sequences from the demultiplexed 16S rRNA gene libraries, following the online MiSeq analysis SOP (http://www.mothur.org.wiki/MiSeqs_SOP). Sequences were firstly merged using the make.contigs command. Reads containing ambiguous bases, homopolymer runs greater than eight bases, and sequences of less than 150 base pairs in length were removed from the dataset. Remaining sequences were aligned against mothur's Silva reference database, after customizing the reference alignment to concentrate on the V4 region only. Further denoising of the dataset was performed using mothur's pre clustering algorithm, allowing for up to two differences between sequences. This sorted sequences by abundance, ordering from most abundant to least and identified sequences within two nucleotides of each other. If sequences met these conditions, they were merged. Chimeric sequences were then removed from the dataset using the UCHIME algorithm in mothur as a final denoising step prior to taxonomic classification.

For taxonomic analyses, sequences were annotated using the Bayesian classifier implemented by the Ribosomal Database Project (RDP) Release 11. A minimum confidence bootstrap threshold of 80% was required for each assignment. Sample coverage, rarefaction curves, bias-corrected Chao 1 richness and Simpson's index of diversity were calculated based on assembled OTU's using mothur. Samples were rarefied to the sample with the lowest number of sequences before performing these diversity analyses, to ensure that any observed differences in diversity were not caused by uneven sampling depth.

4.3.5. Statistical analysis

Statistical analyses of all filtered libraries was conducted according to the mothur MiSeq protocol (Kozich et al 2013). ThetaYC (Yue & Clayton 2005) and Jaccard distance matrices were created within mothur using the dist.shared command. These matrices were calculated to examine the dissimilarity between the microbial community structure and membership of all samples respectively, and take into account the relative abundance of bacterial taxa. Microbial community structure refers to the combination of membership and the abundance of each OTU, whereas microbial community membership refers to the list of OTU's in a community and evaluates their presence/absence. PCoA was performed to visualize the resulting ThetaYC and Jaccard distances. The statistical significance of any observed distances was examined using the Analysis of Molecular Variance (AMOVA) test within the mothur MiSeq analysis protocol. Furthermore, Parsimony (Schloss & Handelsman 2006) and UniFrac (Lozupone & Knight 2005) analyses were performed in order to test whether any observed clustering between samples was statistically significant. Finally, Metastats (White et al 2009) and Indicator (McCune et al 2002) algorithms were used to determine whether any phylotypes were differentially represented between farmed and aquarium rainbow trout intestinal samples. Results were considered as statistically significant at two levels, $p<0.05$ and $p<0.01$. A one-way ANOVA was performed on the Simpson and Chao1 richness data, using Mintab 17 Statistical software (https://www.minitab.com), to test for any significant differences between the mean microbial diversity of the tested trout populations.

4.3.6. Establishment of predicted functional profiles

In the present study, PICRUSt (Langille at al 2013) was used to predict the functional metagenome of all samples. OTU's were firstly picked against the Greengenes v. 13_5 database and the make.biom command within mothur was used in order to produce a file compatible with the PICRUSt program. This BIOM file was uploaded to the online Galaxy terminal

(http://huttenhower.sph.harvard.edu/galaxy/edu) for pre-processing before analysis using the PICRUSt pipeline. PICRUSt was firstly used to correct OTU tables for known 16S rRNA copy numbers for each taxon and then subsequently to predict metagenomes using the precalculated KEGG (Kyoto Encyclopedia of Genes and Genomes) ortholog (KO) and Cluster of Orthologous Genes (COG) tables. Because PICRUSt relies on reference genomes that are phylogenetically similar to those represented in a community, the Nearest Sequenced Taxon Index (NSTI) values were calculated in order to quantify the availability of nearby genome representatives for each microbiome sample, and hence to determine the overall accuracy of the metagenomic predictions for all samples. The output of the PICRUSt analysis consists of a table of quantitative functional counts, i.e. KEGG pathway counts according to sample. Because some KEGG orthologs can be represented in multiple pathways, the categorize_by_function.py command within PICRUSt was used to collapse the functional predictions at the level of the individual pathways. The output files from the PICRUSt analysis were then uploaded to the Statistical Analysis of Metagenomic Profiles (STAMP) (Parks et al 2014) and iPath (Letunic et al 2008, Yamada et al 2011) software packages. These programs permitted the further statistical interrogation of all predicted functional datasets and the production of graphical depictions of key functional pathway data. The iPath tool provides only qualitative data in the determination of the primary overall metabolic and regulatory KEGG pathways identified in the intestinal microbiomes of both rainbow trout populations tested, and was used to create a global map of these pathways.

4.4. Results

4.4.1. Sequencing data and microbial diversity analysis

A total of 14,088,267 reads were obtained from all sample libraries after quality filtering steps were performed. A total of 1131 OTU's were assembled from the combined libraries. After subsampling to the level of the library containing the fewest reads (DF AF1, n=142,267), rarefaction analysis revealed that all sample curves reached saturation (Figure 4.2). Overall, rarefaction estimates pointed to a slightly elevated level of community diversity in the fish farm samples, with the highest level of diversity noted in the aquarium tank biofilm sample. Mean Chao1 richness estimates were higher in the fish farm samples (286.15 ± 125.42) than in the aquarium samples (233.51 ± 90.44), and the estimates were even higher in the tank biofilm sample (691.89), reflecting the trend indicated in the rarefaction analysis. The mean inverse Simpson value was however greater in the aquarium fish samples (2.14 ± 0.78) versus the farm samples (1.64 ± 0.82). However, the overall microbial diversity and richness in the intestine of aquarium and farm based fish were not significantly different (Simpson: f = 3.24, p = 0.088; Chao1: f = 1.14, p = 0.300) (Figure 4.11 S1). Good's coverage estimations were on average >99% for all libraries indicating that a high level of sequence coverage was obtained. All alpha diversity statistics are detailed in Table 4.2.

Table 4.2. Alpha diversity estimates of rainbow trout intestinal microbiomes

Sample	OTU	Coverage	Simpson μ	Simpson σ	Inverse Simpson μ	Inverse Simpson σ	Chao μ	Chao σ
AQ F2	74	0.999802	0.76	0.0005	1.31	0.0009	151.12	5.44
AQ F5	61	0.999814	0.91	0.0003	1.09	0.0004	161.59	6.56
AQ F6	84	0.999797	0.43	0.0004	2.31	0.0023	142.60	4.70
AQ F9	274	0.999595	0.38	0.0002	2.62	0.0002	378.39	8.67
AQ F10	77	0.999781	0.31	0.0001	3.23	0.0020	181.61	6.86
AQ F13	257	0.999521	0.30	0.0002	3.30	0.0028	358.18	8.72
AQ F14	129	0.999705	0.60	0.0005	1.65	0.0015	205.30	6.40
AQ F17	114	0.999716	0.53	0.0005	1.89	0.0017	218.81	7.74
AQ F19	225	0.999634	0.51	0.0005	1.94	0.0021	304.04	6.84
Mean	143	0.999707	0.52	0.0003	2.14	0.0015	233.51	6.88
SD	84	0.000104	0.20	0.0001	0.78	0.0008	90.44	1.34
DF AF1	367	0.999536	0.25	0.0003	4.05	0.0032	477.78	9.12
DF AF2	324	0.999553	0.50	0.0005	1.98	0.0023	403.61	7.37
DF AF3	263	0.999629	0.59	0.0006	1.68	0.0017	350.16	7.32
DF AF4	280	0.999599	0.58	0.0005	1.72	0.0016	371.50	7.73
DF AF5	258	0.999615	0.61	0.0006	1.63	0.0016	332.93	6.86
DF AF6	76	0.999818	0.95	0.0002	1.04	0.0003	129.60	3.99
DF BF1	301	0.999595	0.54	0.0006	1.85	0.0020	381.30	6.91
DF BF2	114	0.999711	0.94	0.0003	1.06	0.0003	203.66	6.87
DF BF3	88	0.999765	0.95	0.0002	1.04	0.0003	156.87	5.52
DF BF4	48	0.999885	0.80	0.0005	1.25	0.0007	90.15	3.12
DF BF5	116	0.999725	0.84	0.0004	1.19	0.0007	188.10	6.06
DF BF6	261	0.999520	0.78	0.0005	1.27	0.0009	348.25	8.00
Mean	208	0.999663	0.69	0.0004	1.64	0.0013	286.15	6.57
SD	106	0.000117	0.21	0.0001	0.82	0.0009	125.42	1.68
Biofilm	496	0.999243	0.20	0.0002	5.09	0.0076	691.89	14.52

Normalized mean values (μ) and standard deviations (σ) for the number of OTU's, sample coverage, Simpson index, inverse Simpson index and Chao 1 richness diversity estimators. Normalized values and standard deviations were obtained by random resampling using rarefaction analysis according to the sample possessing the lowest number of reads (DF AF1, n = 142,267). AQ = Aquarium fish samples, DF = Farmed fish samples (where A = Pen A and B = Pen B), Biofilm = Aquarium tank biofilm sample.

a)
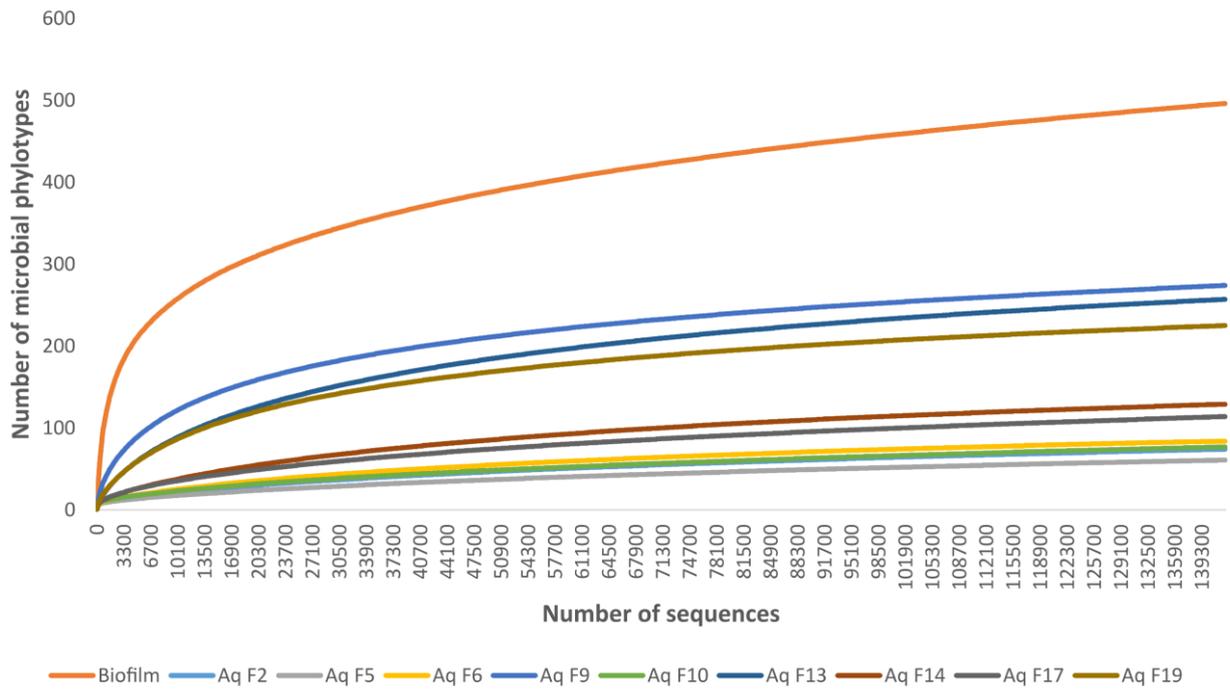
b)
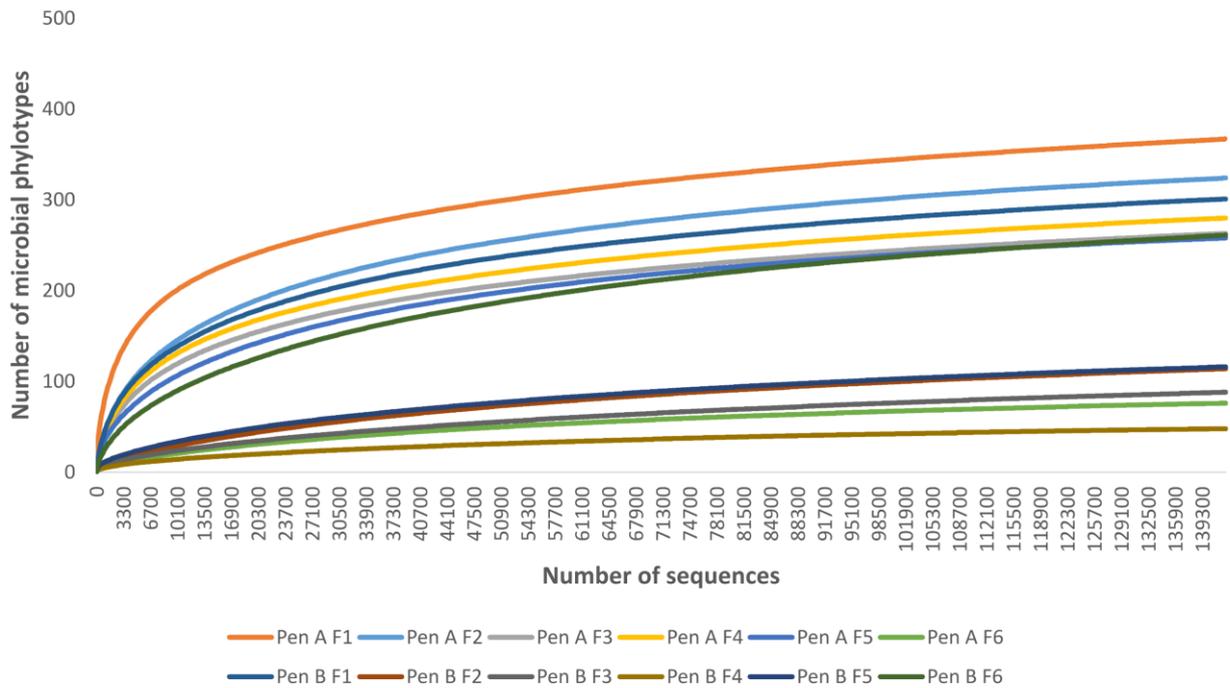
Figure 4.2. Rarefaction analysis of **a)** aquarium and **b)** farm based rainbow trout intestinal microbiome samples. Samples were rarefied according to the library with the lowest number of reads (n = 142267, Sample DF AF1).

4.4.2. Microbiome composition of rainbow trout distal intestine

A total of 14 separate bacterial phyla were observed across all libraries analysed. The mean distribution of OTU's at the phylum level of fish farm, aquarium and biofilm samples is depicted in Figure 4.3. In the fish intestinal samples from both sites, four phyla were dominant, the Tenericutes, Firmicutes, Proteobacteria and Spirochaetae. A total of 18 bacterial classes were recorded within the 14 phyla observed (Figure 4.4). The mean number of OTU's classified to the genus level was 143 (maximum of 274, minimum of 61) and 208 (maximum of 367, minimum of 48) in the aquarium and farmed fish intestine samples respectively.

The Tenericutes were the most dominant phylum in the fish intestinal microbiome samples, from both the aquarium and the fish farm sites. Within this phylum, the Mollicutes were the dominant class and the principal OTU classified at the genus level was *Mycoplasma*. The Mollicutes were slightly more abundant in the farmed fish samples with a mean representation of 81%, versus 68% in the intestine of the aquarium fish. The vast majority of other OTU's belonged to the classes Bacilli, Clostridia, Gammaproteobacteria and Spirochaetia. The remaining 13 classes, Alphaproteobacteria, Betaproteobacteria, Candidate Division WPS-1, Flavobacteria, Fusobacteria, Sphingobacteria, Deinococci, Negativicutes, Actinobacteria, Bacteroidia, Deltaproteobacteria, Thermodesulfobacteria and Opitutae were detected at much lower levels of sequence abundances. The next most prevalent class was the Spirochaetia, with *Brevinema* being identified as the predominant OTU identified. This class was more abundant in the aquarium fish, representing 19.7% versus 8.1% in the farmed fish samples. The phylum Firmicutes was slightly more prominent in the aquarium fish and contained OTU's that were primarily split between two bacterial classes, the Clostridia and the Bacilli. Within these classes, the principal OTU's were identified as *Lactobacillus*, *Acetanaerobacterium*, *Catellicoccus*, *Streptococcus*, *Weissella*, *Leuconostoc*, *Lactococcus*, *Enterococcus* and *Bacillus*. The phylum Proteobacteria was primarily represented by the γ subclass in both the aquarium and the farm based fish samples, with *Photobacterium*, *Pseudomonas*, *Acinetobacter*, *Maricurvus*, *Moritella* and *Pantoea* being the primary genera detected. Members of the α and β subclasses were also recorded, but were poorly represented in the fish intestinal samples.

a)

Tenericutes (68.32% ± 39.97)
Firmicutes (8.43% ± 14.93)
Proteobacteria (2.92% ± 3.48)
Spirochaetes (19.77% ± 29.69)
Bacteroidetes (0.14% ± 0.39)
Verrucomicrobia (0.01% ± 0.01)
Fusobacteria (0.001% ± 0.004)
Actinobacteria (0.01% ± 0.01)
Deinococcus-Thermus (0.002% ± 0.004)
Planctomycetes (0.002% ± 0.003)
Candidate Division WPS-1 (0.35% ± 0.45)
Candidate Division WPS-2 (0.001% ± 0.002)
Chloroflexi (0.0009% ± 0.001)
Cyanobacteria (0.001% ± 0.001)

b)

Tenericutes (81.59% ± 34.54)
Firmicutes (6.89% ± 15.84)
Proteobacteria (1.15% ± 2.91)
Spirochaetes (8.13% ± 27.93)
Bacteroidetes (0.45% ± 1.04)
Verrucomicrobia (0.06% ± 0.14)
Fusobacteria (0.13% ± 0.37)
Actinobacteria (0.04% ± 0.08)
Deinococcus-Thermus (0.003% ± 0.009)
Planctomycetes (0.01% ± 0.02)
Candidate Division WPS-1 (1.46% ± 3.68)
Candidate Division WPS-2 (0.01% ± 0.04)
Chloroflexi (0.005% ± 0.01)
Cyanobacteria (0.02% ± 0.05)

**Figure 4.3.** Mean relative % sequence abundance of microbial phyla recorded in the distal intestine of **a)** aquarium and **b)** farm-based fish.

a)

b)
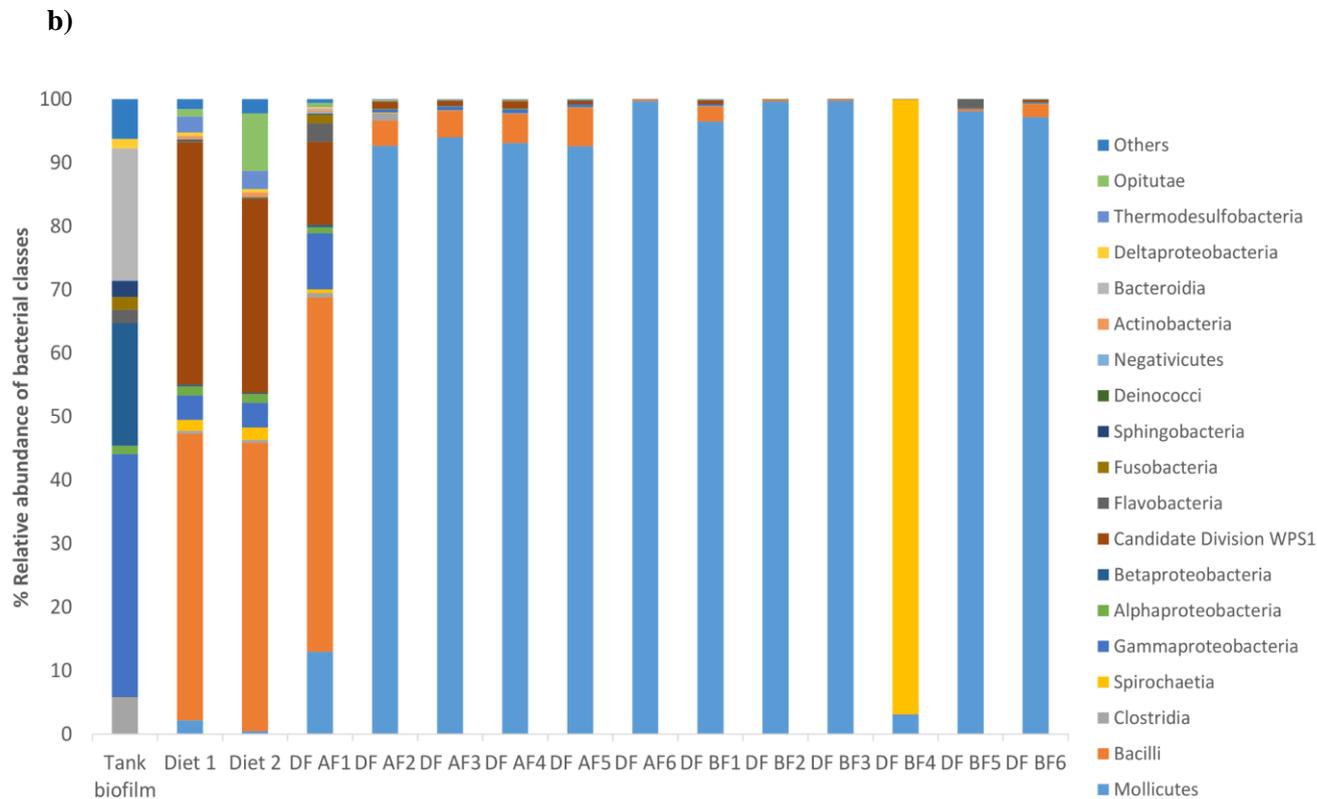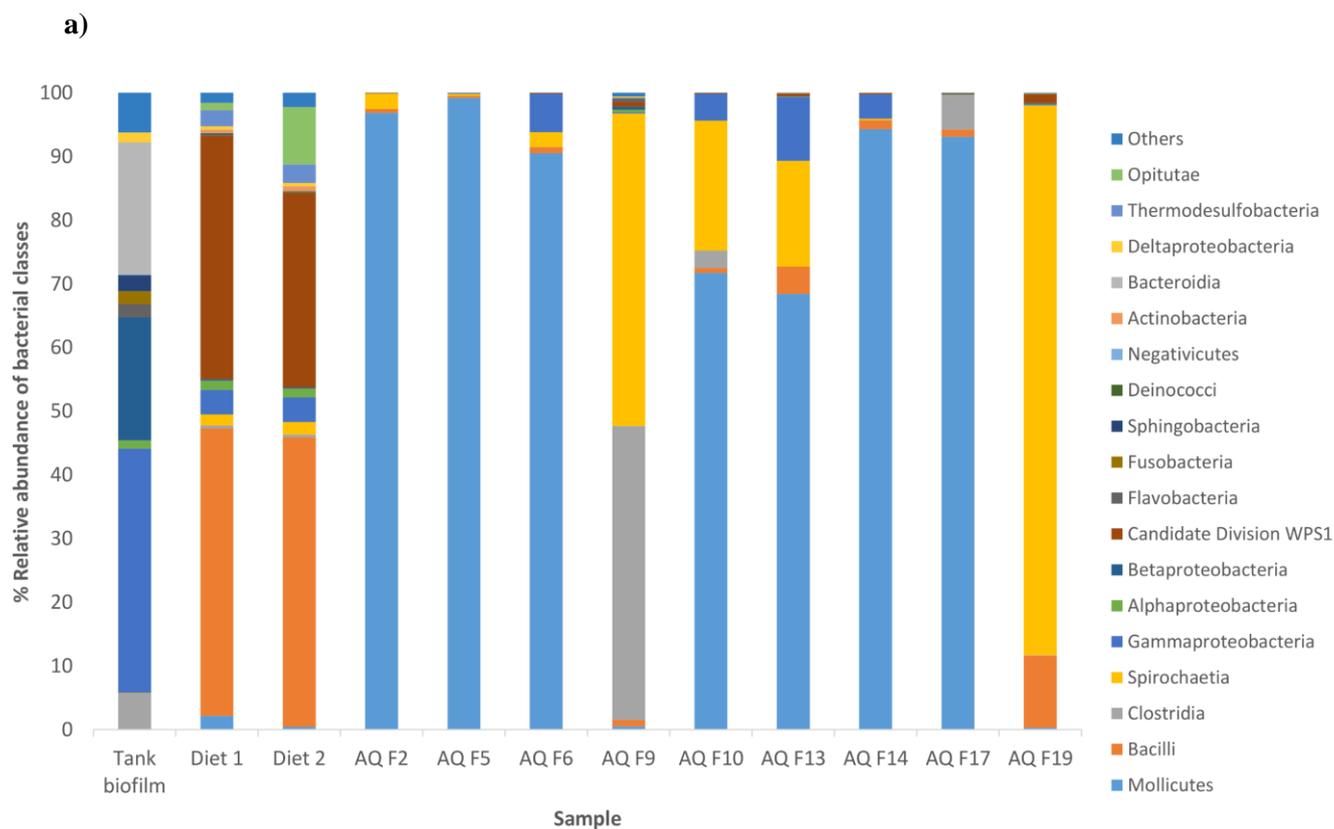
Figure 4.4. Relative % sequence abundance of aquarium tank biofilm, diet and intestinal microbial classes observed in individual fish sampled from **a)** aquarium (n=9) and **b)** farm (n = 12).

4.4.3. Microbiome composition of aquarium tank biofilm and diets

In contrast to the rainbow trout intestinal samples, the tank biofilm sample was dominated by members of the Proteobacteria and Bacteroidetes, whilst the remaining OTU's were largely composed of members of the Firmicutes and Fusobacteria. The primary phylotypes within the Proteobacteria belonged to the γ and β subclasses with the most numerous OTU's being identified as *Acidiferrobacter*, *Sedimenticola*, *Arenicella*, *Sphaerotilus*, *Polaromonas*, *Albidiferax* and *Undibacterium*. The phylum Bacteroidetes was principally represented by OTU's belonging to the class Bacteroidia with *Alkalitalea*, *Paludibacter* and *Flectobacillus* being the chief genera detected. The phylum Firmicutes was largely composed of a single OTU of the class Clostridia, identified as *Clostridium sensu stricto*. *Propionigenium* was the primary OTU assigned to the Fusobacteriaceae recorded in the tank biofilm library. The microbiome of the diet pellets was dominated by the phylum Firmicutes (mean sequence abundance 45%) and Candidate Division WPS-1 (mean sequence abundance 34%). Of the Firmicutes, the class Bacilli was well represented, with *Lactobacillus* dominating the sequence libraries in both of the diet pellet samples that were tested.

4.4.4. Statistical analyses

Two separate distance matrices, ThetaYC and Jaccard, were computed in order to compare the structure and membership of the intestinal microbial communities between the two rainbow trout populations sampled. PCoA of the first and second axes of the ThetaYC distances (69% of the total variation) suggested that the microbial community structure between both fish populations was similar, with both sample sets clustering close together (Figure 4.5a, Figure 4.12 S2a). The AMOVA analysis confirmed that any spatial separation observed in the PCoA of ThetaYC distances was not statistically different between the aquarium and farmed trout (F_s = 1.20, p = 0.292). Furthermore, Parsimony and UniFrac tests were in agreement with the AMOVA result (ParsSig = 0.085, UWSig = 0.26). The microbiome structure of the biofilm sample was however significantly different from the fish intestinal samples (UWSig = <0.001). In addition, the microbiome of the diet pellets was also found to be significantly different from the intestinal samples (WSig = <0.001, UWSig = 0.003, ParsSig = 0.025).

The Jaccard distance matrix, a further measure of dissimilarity between communities, was calculated to compare the community membership of the samples (Figure 4.5b, Figure 4.12 S2b). A slight separation in the clustering of both fish populations was observed in the PCoA plots created from this distance matrix. When an AMOVA was performed on this Jaccard

135

matrix, the spatial separation was established as being statistically significant. (F_s = 2.41, p = 0.001). The Parsimony (ParsSig = <0.001) and UniFrac tests (WScore = 0.894, WSig = <0.001, UWScore = 0.981, UWSig = <0.001) confirmed this result, indicating that the microbial community membership was significantly different between the farmed and the aquarium fish. In addition, the tank biofilm sample was significantly different, in terms of community membership, from the farmed fish samples (AMOVA F_s = 1.97, p = 0.003), but not from the aquarium fish samples (AMOVA F_s = 1.70, p = 0.096) when clustering from the PCoA was analysed.

Metastats and Indicator analyses revealed that a number of genera were discriminatory according to farming environment (Table 4.3). The genera *Photobacterium*, *Catellicoccus*, *Moritella*, *Ureibacillus*, *Paralactobacillus*, *Psychrilyobacter*, *Thermobacillus*, *Lactobacillus* and *Fusobacterium* were all discriminatory with the farm based fish and they were significantly more abundant in these individuals. In addition, the genera *Sphaerotilus*, *Maricurvus* and *Weissella* were differentially represented in the aquarium fish (Figure 4.6a, b).

a)
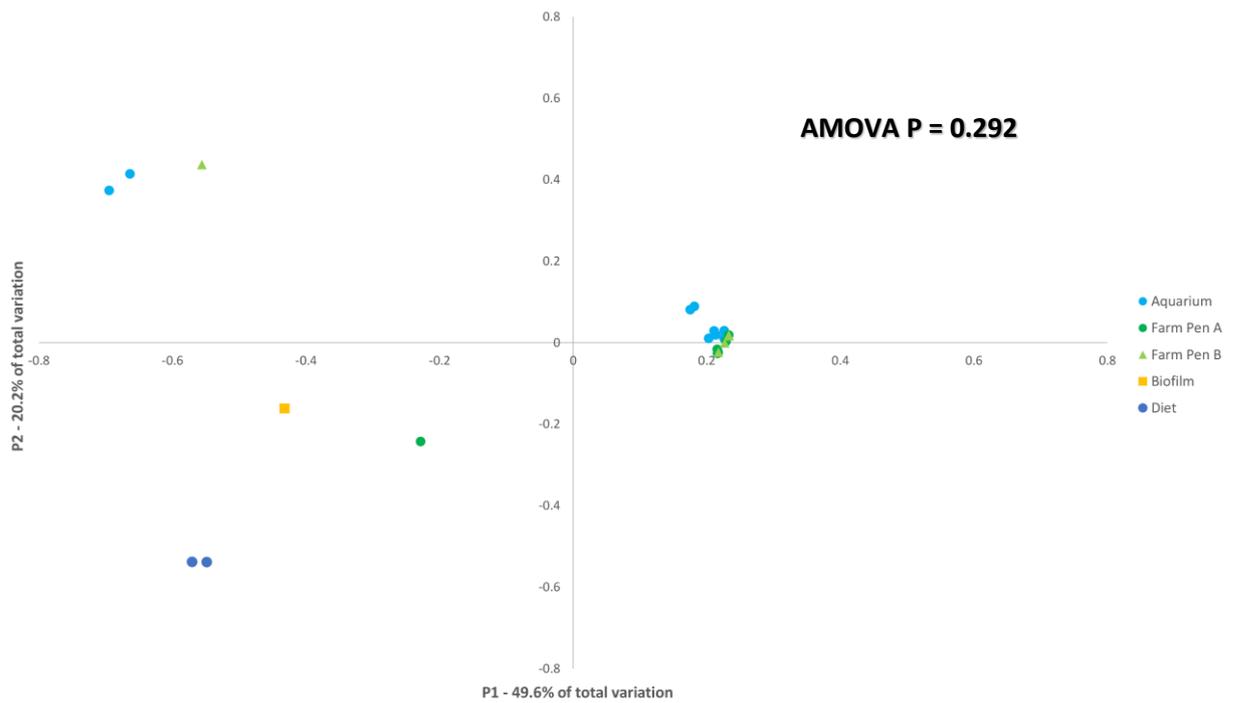
b)
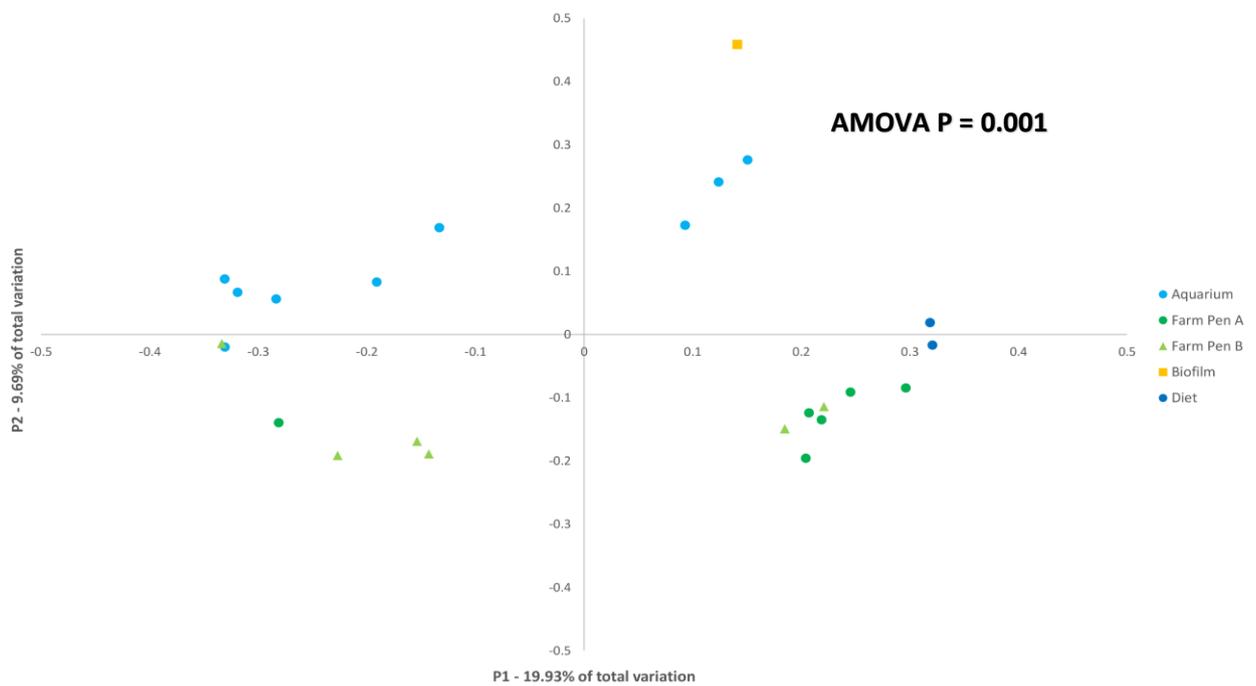
Figure 4.5. Principal coordinate analysis (PCoA) depicting differences in microbial community structure and membership between aquarium fish, farm-based fish, tank biofilm and diet pellet samples based on **a)** ThetaYC and **b)** Jaccard distances respectively. Each dot represents an individual sample.

a)
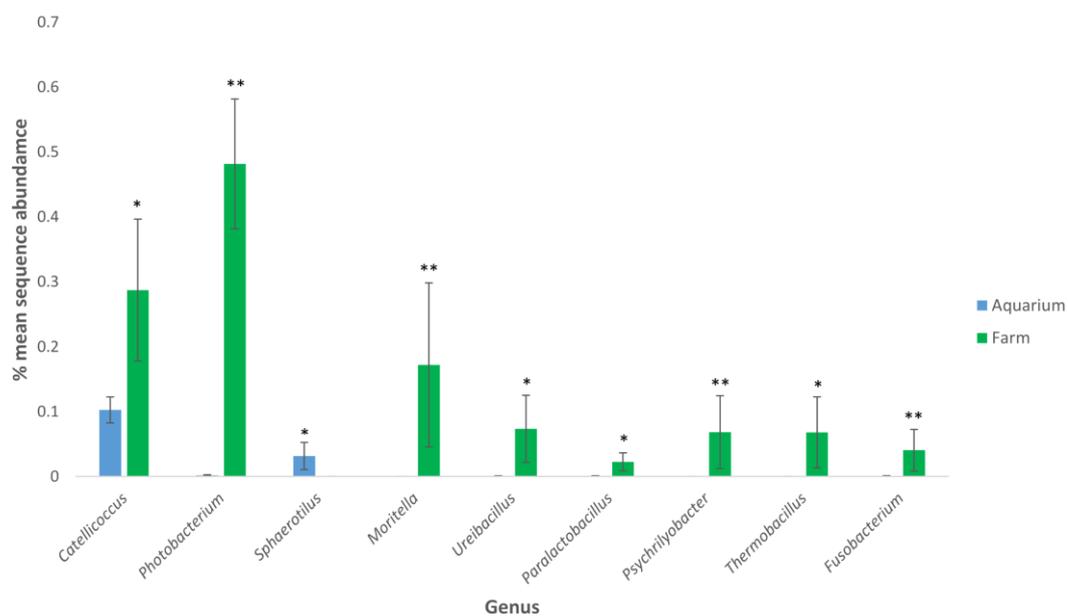
b)
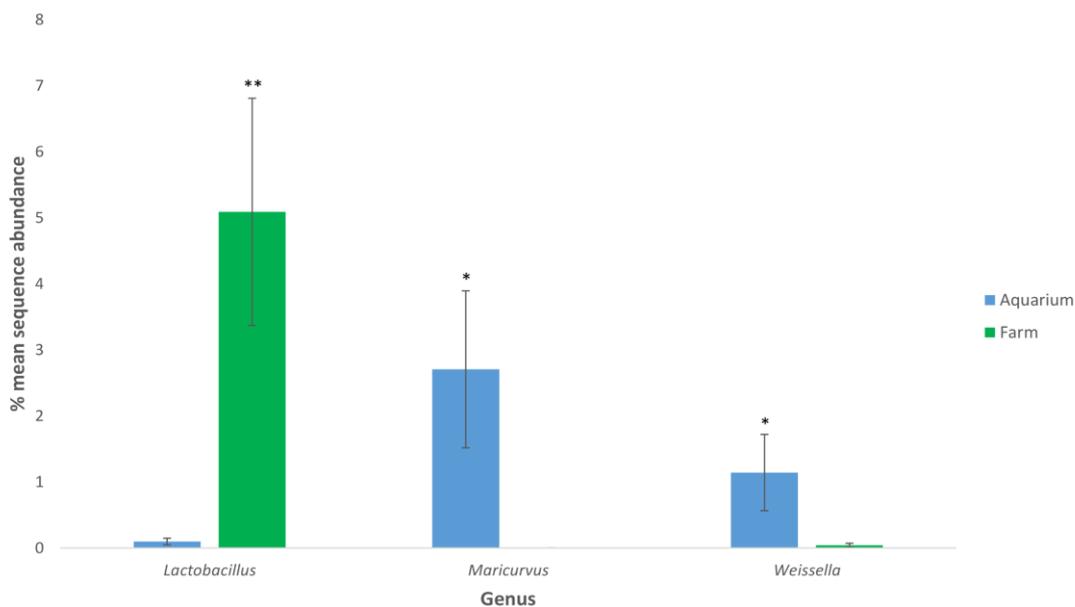
Figure 4.6. Bacterial taxa identified by Metastats and Indicator analysis as discriminatory between aquarium and farm based rainbow trout intestinal samples. The data are plotted as mean relative percentage sequence abundance ± SEM *P<0.05 **P<0.01. Data are split into **a** and **b** to improve interpretation.

Table 4.3. **Phylotypes identified as discriminatory according to rearing environment by both Metastats and Indicator analyses. Statistical significance was accepted on two levels (p<0.05, p<0.01**)

Phylotype	p value		Discriminator
	Metastats	**Indicator**	**Discriminator**
Photobacterium	0.0009	0.0009	Farm
Catellicoccus	0.001	0.034	Farm
Moritella	0.0009	0.0009	Farm
Ureibacillus	0.001	0.014	Farm
Paralactobacillus	0.0009	0.007	Farm
Psychrilyobacter	0.0009	0.002	Farm
Thermobacillus	0.0009	0.044	Farm
Lactobacillus	0.0009	0.006	Farm
Fusobacterium	0.0009	0.0009	Farm
Maricurvus	0.001	0.049	Aquarium
Weissella	0.0009	0.003	Aquarium
Sphaerotilus	0.001	0.031	Aquarium

4.4.5. Predicted functional metagenomes of the rainbow trout intestinal microbiome

PICRUSt was used to predict the functional potential of the intestinal microbiome of rainbow trout. Mean NSTI values were 0.114 ± 0.157 and 0.064 ± 0.116 for the aquarium and farm samples respectively (Table 4.4), indicating that all samples were tractable for PICRUSt analysis (Langille et al 2013). KEGG orthologs were classified to level 3. The majority of the predicted functional pathways were found to belong to four main categories. These were as follows: 1) metabolism 2) environmental information processing 3) genetic information processing and 4) cellular processes (Figure 4.7). No significant differences were noted in predicted functional potential between both populations of fish sampled (Figure 4.8). Within the metabolism pathways, increases in genes associated with carbohydrate, protein and amino acid metabolism were noted, and to a lesser extent pathways associated with energy, vitamin and lipid metabolism. The environmental information processing category was dominated by genes associated with membrane transport and signal transduction. Genes associated with transporters, ABC transporters, the bacterial secretion system, the phosphotransferase system and the two component system were identified. Genetic information processing pathways contained genes involved in protein folding and export, transcription, translation, and DNA replication and repair. The iPath data correlated with the output from PICRUSt and STAMP in that pathways relating to amino acid, carbohydrate, nucleotide and energy metabolism were the most abundant metabolic pathways present in both populations of fish, when visualized with this qualitative tool (Figure 4.9). Finally, cellular motility factors, bacterial chemotaxis and flagellar assembly were predominant in the cellular processes KEGG regulatory category, in addition to membrane transport, translation and signal transduction pathways (Figure 4.10).

Table 4.4. Weighted Nearest Sequence Taxon Index (NSTI) values for predicted functional metagenomes of rainbow trout intestinal microbiomes (AQ=Aquarium fish, DF=Farm-based fish)

Sample	Weighted NSTI
AQ F2	0.023
AQ F5	0.019
AQ F6	0.025
AQ F9	0.364
AQ F10	0.072
AQ F13	0.066
AQ F14	0.020
AQ F17	0.021
AQ F19	0.411
Mean	0.114
SD	0.157
DF AF1	0.157
DF AF2	0.024
DF AF3	0.022
DF AF4	0.023
DF AF5	0.021
DF AF6	0.018
DF BF1	0.020
DF BF2	0.018
DF BF3	0.018
DF BF4	0.412
DF BF5	0.018
DF BF6	0.020
Mean	0.064
SD	0.116
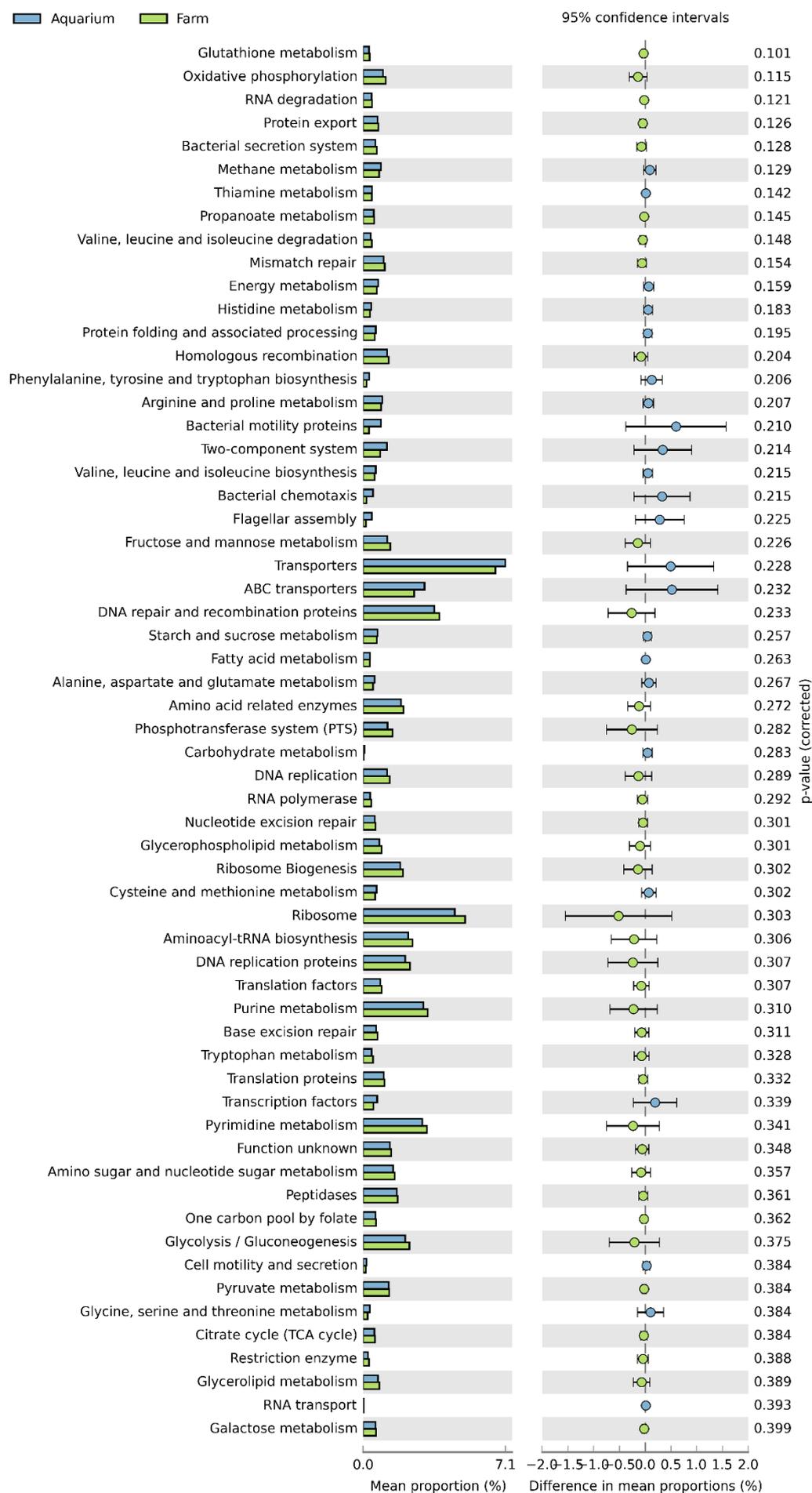
Figure. 4.7. Predicted functional metagenomic pathways of rainbow trout intestinal microbiome, as identified by PICRUSt and STAMP analyses.
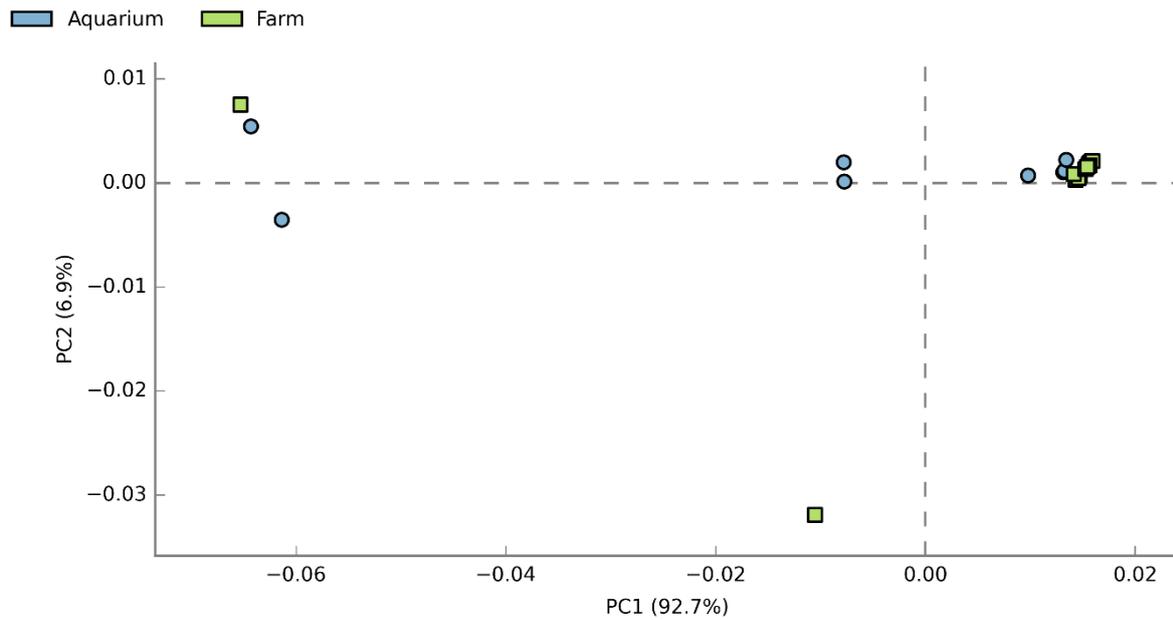
Figure 4.8. Principal coordinate analysis (PCoA) of predicted functional metagenomes between intestinal microbiomes of aquarium and farm-based rainbow trout. Each dot represents an individual sample.

a)

b)

Figure 4.9. KEGG metabolic pathways of functions encoded by the rainbow trout intestinal microbiome from **a)** aquarium and **b)** farm reared fish (iPath).
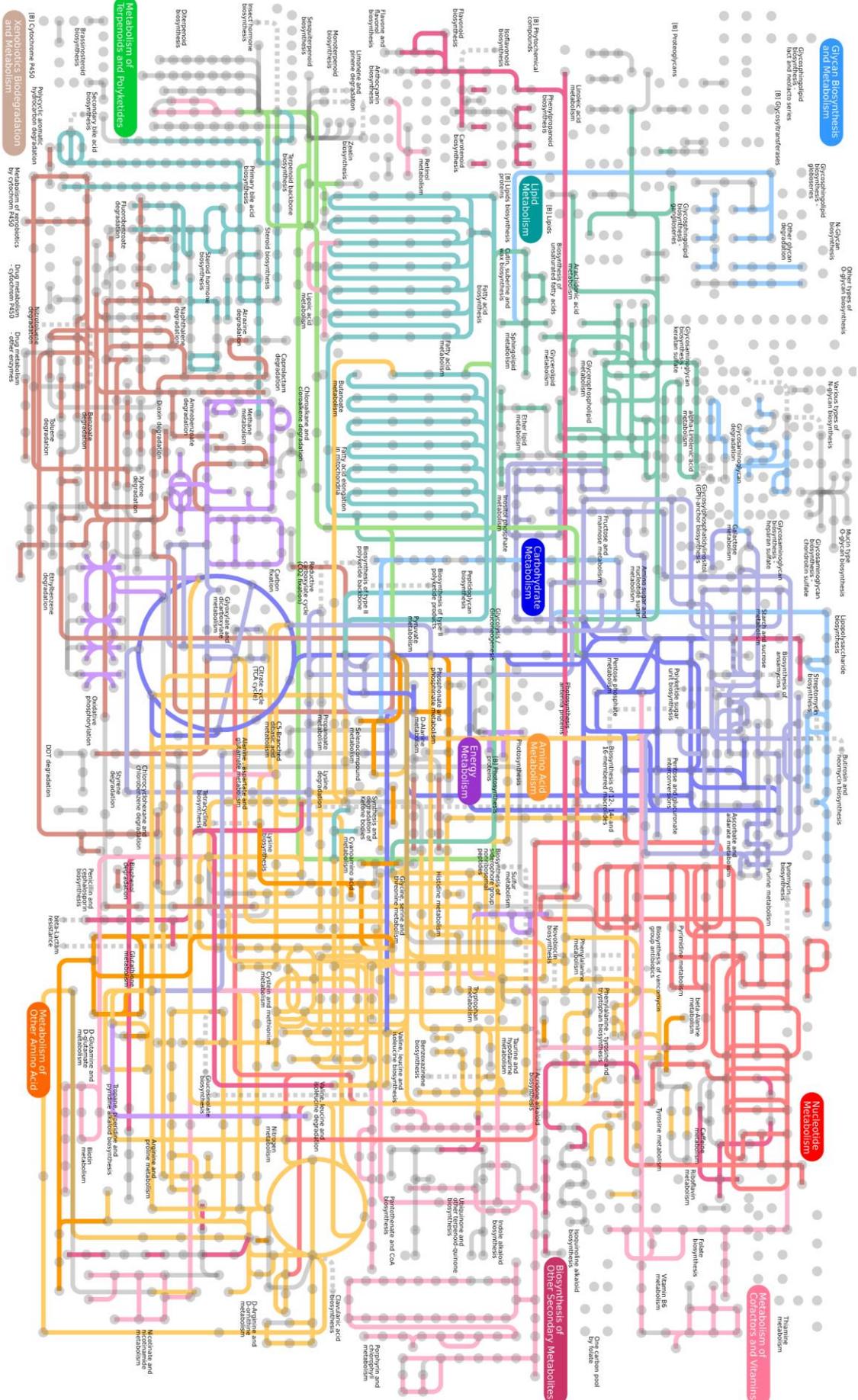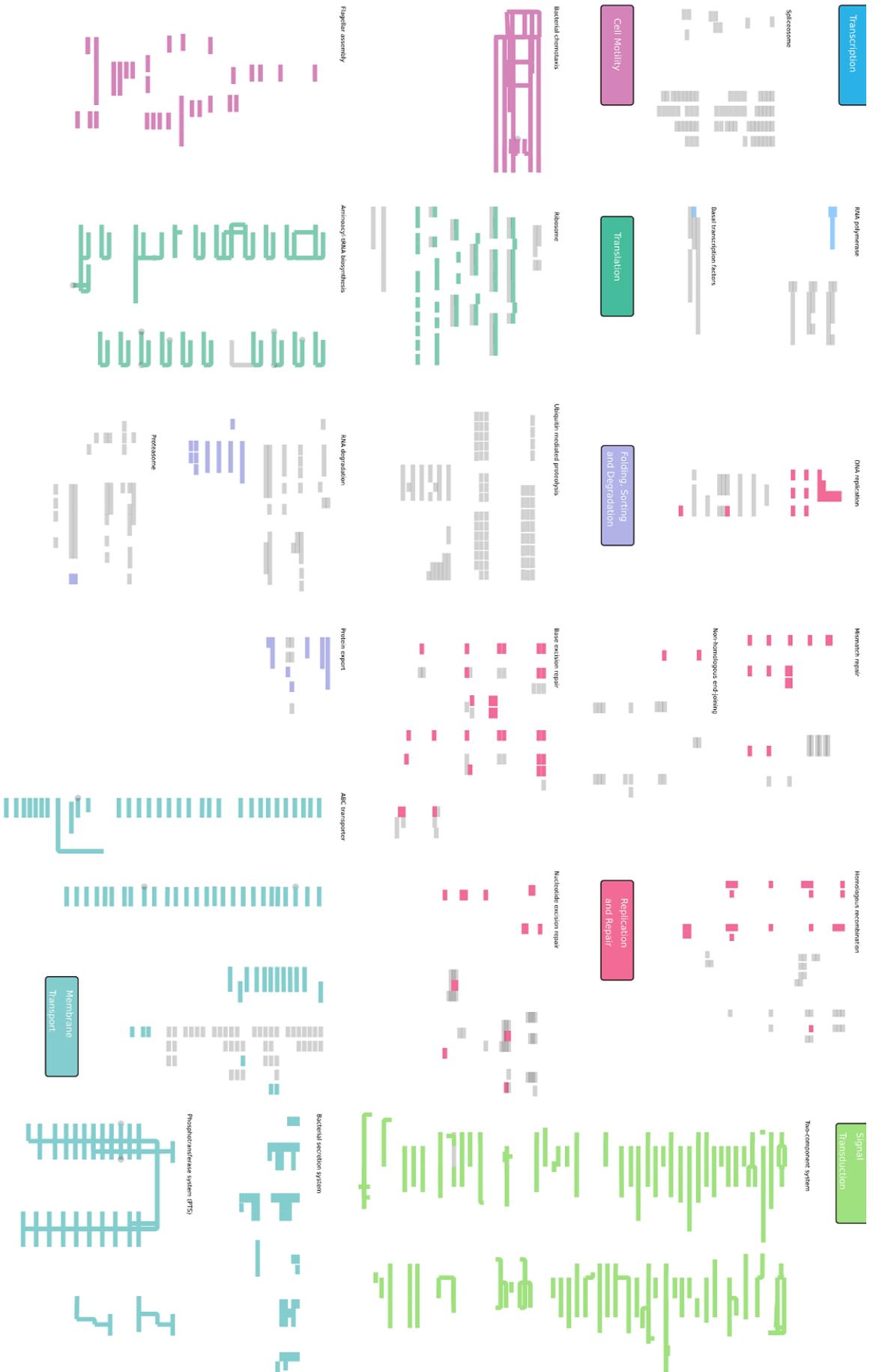
Figure 4.10. KEGG regulatory pathways of functions encoded by the rainbow trout intestinal microbiome from all fish sampled in this study (iPath).

4.5. Discussion

The geographical location of fish farms has been posited to have an impact upon the composition of the intestinal microbiome of the cultured individuals, due to the influence of the native microbial ecology of each site (Ringø et al 1995, Holben et al 2002, Lozupone & Knight 2007, Sullam et al 2012, Giatsis et al 2015). To date, most studies have focused on establishing the diversity and stability of salmonid gut microbiomes from single aquaculture facilities (Wong et al 2013, Zarkasi et al 2014). Furthermore, there is a paucity of information relating to the functional potential of these bacteria and how they might influence the overall health of the fish. The study reported here is, to the author's knowledge, the first to employ high throughput sequencing methods to characterize the phylogeny and functionality of the intestinal microbiome of rainbow trout at two different rearing locations. The results of this research have revealed that the overall structure of the microbiome between the farm and aquarium raised fish analysed in this study was very similar, however the community membership was significantly different between the two populations. The data generated using PICRUSt revealed that the predicted functional potential of these communities was similar between both groups, and suggests that these communities might play an active role in the metabolism of dietary ingredients.

Phylum level assignment of OTU's indicated a dominance of Tenericutes among all of the fish sampled from both locations. The genus *Mycoplasma* was especially prevalent in all of the rainbow trout intestinal libraries analysed in this study. The phylum Tenericutes was also present in the aquarium biofilm and diet samples tested, but at very low levels of detection when compared with the fish intestinal samples, suggesting that members of this phylum might be specifically adapted to the gastrointestinal environment of farmed rainbow trout. However, further samples of the tank/cage biofilm and diet pellets would need to be collected and analysed from both environments in future studies in order to confirm this hypothesis. *Mycoplasma* were first reported to be a major component of the intestinal microbiome of wild Atlantic salmon (Holben et al 2002) and then the Californian mudsucker (Bano et al 2007) and have since been observed in the GI tract other fish and shellfish species (Moran et al 2005, Kim et al 2007, King et al 2012). An increasing number of studies are currently revealing its dominance within the intestine of farmed salmonids (Abid et al 2013, Green et al 2013, Zarkasi et al 2014, Llewellyn et al 2015, Lowrey et al 2015, Ozório et al 2015) and yet its function within the GI tract of these fish remains poorly understood. The prevalence of this phylotype in both the aquarium and farm-based fish may suggest that the geographical location of the

rearing environment does not impact upon its presence, and that rainbow trout could be a specific host for this microbe. The Mycoplasmataceae are fastidious organisms, and are difficult to grow on conventional microbiological isolation media. This might explain why their abundance in the rainbow trout intestine is now being reported more frequently, as studies that employ high throughput sequencing methods are published.

There was no significant difference in mean microbial diversity between the farm and the aquarium samples. A higher diversity in the farm samples was initially expected, given that the aquarium based fish were maintained in a single aquaculture facility, in flow-through tanks without water recirculation. Furthermore, the aquarium reared trout were obtained from a single supplier and from the same egg source. These combined factors would likely have limited the environmental variation and may have increased the probability of a similar microbiome structure and membership. In contrast, the farm samples were obtained from cages situated in a Scottish loch, and hence these fish were more likely to have been exposed to a greater diversity of microorganisms. However, the mean microbial diversity, whilst slightly higher in the farm samples, was remarkably similar between both populations in spite of the different environmental conditions of each site. This suggests that other factors aside from the geographical location of the culture system may be more influential drivers of microbial diversity in the rainbow trout intestine.

Some studies of the intestinal microflora of rainbow trout have hypothesized that the composition could mirror that of the surrounding aquatic environment (Trust & Sparrow 1974, Yoshimizu & Kimura 1976, Sugita et al 1982, Ringø & Strom 1994, Nayak 2010, Semova et al 2012, Xing et al 2013, Sullam et al 2015). However, the microbiome structures of the aquarium tank biofilm and the diet samples were significantly different from the intestinal libraries in the present study. These data suggest that the intestinal microbiome may be specialized, and may not simply be a reflection of the microbial flora of the surrounding environment. Future studies should include analyses of the microbiome of the farm and aquarium water in order to further explore this theory. The addition of such a sample to future studies would also aid in comparing the relative functional potential between commensal bacteria within the rainbow trout intestine and those of their rearing environment, and may help to elucidate the primary functional traits of most importance to rainbow trout physiology. The PCoA revealed a homogeneity between the structure of the intestinal microbiome in the farm and aquarium based fish. However, the community membership was significantly different between the groups. This suggests that the 'core' microbial phyla and classes are somewhat

stable in the rainbow trout intestine, regardless of geographical location, but that other assemblages of more sporadic OTU's can vary accordingly. These results reflect those reported in similar studies. Roeselers et al (2011) revealed that individual zebrafish (*Danio rerio*), sampled from wild and domesticated populations, shared a stable core gut microbiome independent of their origin. Another recent study on the wild Atlantic salmon intestinal microbiome found that community composition was not significantly impacted by geography and that individual fish, at different life stages, possessed remarkably similar intestinal microbiome structures which were distinct from those found in the environment (Llewellyn et al 2015). Furthermore, Bakke et al (2015) reported that cod (*Gadhus morhua*) larvae shared a gut microbiome structure significantly different to that of their rearing water and diet. Taken together, these findings are suggestive of specialized and potentially co-evolved associations between fish species and their intestinal microbiota.

The presence of a number of OTU's that were discriminatory according to geographical location most likely explains the spatial separation observed in the community membership plots. The genus *Lactobacillus* was significantly more abundant in the farm based fish. Its elevated levels suggest that this organism may have been enriched by the diet, possibly as it is known that the relative abundance of this bacterium is affected by diet type (Desai et al 2012, Wong et al 2013, Ingerslev et al 2014), and both populations of fish sampled in this study were fed different diets. Lactobacilli are commonly observed inhabitants of the teleost fish gut, but usually represent a minor proportion of the overall microbial community (Desai et al 2012, Merrifield et al 2014). Other organisms such as *Moritella*, *Photobacterium* and *Psychrilyobacter* were also found to be discriminatory according to location, and were significantly more abundant in the farm raised fish. The exact reason for this is unclear, but some species of *Moritella* and *Photobacterium* are fish pathogens known to cause conditions such as winter ulcer disease and pasteurellosis respectively (Fouz et al 1992, Gauthier et al 1995, Lunder et al 1995, Pedersen at al 1997, Benediktsdottir et al 1998, Bruno et al 1998, Lovoll et al 2009) in farmed salmonids, and all three of these genera are primarily associated with cold water temperatures. It should be noted that at the time of sampling, the fish farm was experiencing the lowest average water temperature recorded for that calendar month in over a decade. This could perhaps explain the enrichment of these psychrophilic bacterial taxa within the intestine of these particular fish.

The principal functional pathways expressed in both populations of fish were primarily associated with metabolism, transport and cellular processes and the predicted core functional

potential of the intestinal microbiome was similar in both of the trout populations examined. Membrane transport pathways, such as ABC transporters, utilize the energy of ATP binding and hydrolysis to transport substrates across cellular membranes (Rees et al 2009). They are essential to cell viability and growth and therefore vital for bacterial survival in the intestinal ecosystem. Genes affiliated with the phosphotransferase system (PTS) were found to be abundant in the intestinal microbiomes of both farmed and aquarium reared trout, and this system is used by bacteria for sugar uptake where the source of energy is from phosphoenolpyruvate (PEP), a key intermediate in glycolysis (Meadow et al 1985, Erni 2012). The PTS is a multicomponent network that always involves enzymes of the both the plasma membrane and the cytoplasm, and is involved in transporting many different sugars into bacterial cells, including glucose, mannose, fructose and cellobiose. Two component system pathways, that are commonly found in all prokaryotes, were also enhanced, and modulate gene expression based on environmental stimuli such as temperature, pH and nutrient availability (Mitrophanov & Groisman 2008). The upregulation of these gene pathways suggests that the intestinal microbiome could play an active role in sensing and utilizing sugars as resources for energy production and for the biosynthesis of cellular components.

It is well documented that rainbow trout exhibit poor utilization of dietary carbohydrates (Lovell 1989, Guillaume & Choubert 1999, Geurden et al 2014), but the precise reasons for this remain unclear. The involvement of gene pathways dictating carbohydrate metabolism suggests that members of the microbiome may actively carry out fermentative processes within the intestine. Members of the phylum Firmicutes and Spirochaetes are known to play important roles in the fermentation of dietary carbohydrates, transporting non-digestible sugars across their cellular membranes (Corrigan et al 2015). For most microbial fermentations, glucose dissimilation occurs through the glycolytic pathway. The most commonly produced molecule from this process is pyruvate. Therefore, the enhanced glycolysis/gluconeogenesis and pyruvate metabolism pathways represent a further indication of the fermentative potential of the intestinal microbiome of trout, and may be correlated with the presence of Firmicutes as one of the core microbial phyla observed in the rainbow trout intestine. The fermentation of dietary carbohydrate by members of the intestinal microbiota results in the formation of SCFA such as acetate, propionate and butyrate, which can be utilized in energy metabolism and which have also been shown to promote the health of intestinal enterocytes (Hamer et al 2008, Louis & Flint 2009). The ability of the rainbow trout intestinal microbiome to utilize dietary

carbohydrate as an energy yielding substrate is thus an interesting avenue for future research, and may improve our understanding of carbohydrate digestibility in fish.

The elevation of gene pathways responsible for amino acid fermentation and peptidase production could be linked to the high protein nature of rainbow trout aquafeeds. Rainbow trout require high levels of dietary protein, i.e. more than 35% of diet dry matter (National Research Council 2011). This is most likely linked to persistent amino acid catabolism for their use as an energy source (Kaushik & Seiliez 2010, Geurden et al 2014). Dietary proteins that escape digestion by key endogenous digestive enzymes such as chymotrypsin and trypsin are made available to bacteria for fermentation. These enzymes originate in the pancreas and are not produced by the intestine itself (Guillaume & Choubert 1999). Therefore, the fermentative activity of the microbiome may be particularly important in the distal intestinal region, where such enzymes are likely to be less influential. The Clostridia were abundant in all fish, and are recognized as being proteolytic bacteria that can ferment amino acids (Neis et al 2015). The amino sugar metabolic pathway, expressed by the intestinal microbiome of the fish in this study, is specifically responsible for breaking down protein into its constituent di- and tri-peptides and amino acids (Miska et al 2014, Shaufi et al 2015). These can then be utilized in energy metabolism, used to form the structural components of intestinal epithelial cells or exported to the liver for further processing. There is evidence that symbiotic intestinal microbes of other animals manufacture peptidases and amino acids that are then provided to the host (Douglas 2013, Neis et al 2015). Moreover, Clements et al (2014) recently speculated on the involvement of the intestinal microbiome of fish in protein metabolism, and Kuz'mina et al (2015) demonstrated that the intestinal microflora of crucian carp contributed ~45% of total peptidase production in this species. Additionally, Zarkasi et al (2016) reported a progressive enrichment of proteolytic bacteria in the distal intestine of cage farmed Atlantic salmon, concurrent with increasing levels of dietary protein inclusion. The metagenomic data indicate that similar microbially mediated mechanisms of protein breakdown may occur in the rainbow trout intestinal tract, which could supplement the action of endogenous digestive enzymes. Protein fermentation pathways, similar to those for carbohydrate fermentation, can also result in the production of SCFA, especially branched chain fatty acids (BCFA), which can then be metabolized by the host (Jha & Berrocoso 2016).

In summary, the results show that the core microbiome structure between the two populations of rainbow trout remained similar, regardless of the differences in their rearing environment. Five bacterial phyla, the Tenericutes, Firmicutes, Spirochaetes, Proteobacteria and

Bacteroidetes were dominant in all of the fish intestine samples. The Tenericutes, and in particular, the genus *Mycoplasma* was the most dominant genus in all read libraries. The pattern of dominance of this microbe, in conjunction with its streamlined genome, is suggestive of an obligate symbiotic relationship with the rainbow trout intestine. No significant differences were observed in microbial community diversity or structure between both groups, indicating that the overall composition of the rainbow trout intestinal microbiome may be conserved irrespective of the location of the farming system. Significant differences in community membership were however observed, which suggests that more sporadic taxa unique to each environment may successfully inhabit the intestinal tract of the trout. The functional data obtained in this study demonstrate that the rainbow trout intestinal microbiome possesses the capability to influence protein and carbohydrate metabolism, and may therefore complement the action of endogenous digestive enzymes. Future studies should focus on the profiling of metabolites from pathways identified by functional metagenomics, in order to further evaluate the overall contribution of these microbes to the digestive and energetic processes of farmed fish. Such additional research will enhance our ability to exploit the functional potential of the intestinal microbiome, and could aid in the development of novel nutritional strategies that improve the gut health of rainbow trout.

4.6. Acknowledgements

4.7. Conflicts of interest

The authors declare no conflicts of interest.

4.8. References

Abid, A., Davies, S.J., Waines, P., Emery, M., Castex, M., Gioacchini, G., Carnevali, O., Bickerdike, R., Romero, J. and Merrifield, D.L. (2013) Dietary synbiotic application modulates Atlantic salmon (*Salmo salar*) intestinal microbial communities and intestinal immunity. *Fish & Shellfish Immunology* **35**, 1948-1956

Andrews, S. (2010) FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Bakke, I., Coward, E., Andersen, T. and Vadstein, O. (2015). Selection in the host structures the microbiota associated with developing cod larvae (*Gadhus morhua*). *Environmental Microbiology* **17**, 3914-3924

Bano, N., Derae Smith, A., Bennett, W., Vasquez, L. and Hollibaugh, J.T. (2007) Dominance of *Mycoplasma* in the guts of the long-jawed mudsucker, *Gillichthys mirabilis*, from five California salt marshes. *Environmental Microbiology* **9**, 2636-2641

Benediktsdottir, E., Helgason, S. and Sigurjonsdottir, H. (1998) *Vibrio* spp. isolated from salmonids with shallow skin lesions and reared at low temperature. *Journal of Fish Diseases* **21**, 19-28

Bruno, D.W., Griffiths, J., Petrie, J. and Hastings, T.S. (1998) *Vibrio viscosus* in farmed Atlantic salmon *Salmo salar* in Scotland: field and experimental observations. *Diseases of Aquatic Organisms* **34**, 161-166

Cahill, M.M. (1990) Bacterial flora of fishes: A review. *Microbial Ecology* **19**, 21-41

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G. and Knight, R. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal* **6**, 1621-1624

Carda-Dieguez, M., Mira, A. and Fouz, B. (2014) Pyrosequencing survey of intestinal microbiota diversity in cultured sea bass (*Dicentrarchus labrax*) fed functional diets. *FEMS Microbiology Ecology* **87**, 451-459

Clements, K.D., Angert, E.R., Montgomery, W.L. and Choat, J.H. (2014) Intestinal microbiota in fishes: what's known and what's not. *Molecular Ecology* **23**, 1891-1898

Corrigan, A., De Leeuw, M., Penaud-Frezet, S., Dimova, D. and Murphy, R.A. (2015) Phylogenetic and functional alterations in bacterial community compositions in broiler ceca as a result of mannan oligosaccharide supplementation. *Applied and Environmental Microbiology* **81**, 3460-3470

Desai, A.R., Links, M.G., Collins, S.A., Mansfield, G.S., Drew, M.D., Van Kessel, A.G. and Hill, J.E. (2012) Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **350**, 134-142

Douglas, A.E. (2013) Microbial brokers of insect-plant interactions revisited. *Journal of Chemical Ecology* **39**, 952-961

Erni, B. (2012) The bacterial phosphoenolpyruvate: sugar phosphotransferase system (PTS): an interface between energy and signal transduction. *Journal of the Iranian Chemical Society* **10**, 593-630

Fouz, B., Larsen, J.L., Nielsen, B., Barja, J.L. and Toranzo, A.E. (1992) Characterization of *Vibrio damsela* strains isolated from turbot *Scophthalmus maximus* in Spain. *Diseases of Aquatic Organisms* **12**, 155-156

Gauthier, G., Lafay, B., Ruimy, R., Breittmayer, V., Nicholas, J.L., Gauthier, M. and Christen, R. (1995) Small sub-unit rRNA sequences and whole DNA relatedness concur for the reassignment of *Pasteurella piscicida* (Snieszko et al.) Janssen and Surgalla to the genus *Photobacterium* as *Photobacterium damsela* subsp. *piscicida* comb. nov. *International Journal of Systematic Bacteriology* **45**, 139-144

Geurden, I., Mennigen, J., Plagnes-Juan, E., Veron, V., Cerezo, T., Mazurais, D., Zambonino-Infante, J., Gatesoupe, J., Skiba-Cassy, S. and Panserat, S. (2014) High or low dietary carbohydrate:protein ratios during first-feeding affect glucose metabolism and intestinal microbiota in juvenile rainbow trout. *The Journal of Experimental Biology* **217**, 3396-3406

Ghanbari, M., Kneifel, W. and Domig, K. (2015) A new view of the fish gut microbiome: Advances from next-generation sequencing. *Aquaculture* **448**, 464-475

Giatsis, C., Sipkema, D., Smidt, H., Heilig, H., Benvenuti, G., Verreth, J. and Verdegem, M. (2015) The impact of rearing environment on the development of gut microbiota in tilapia larvae. *Scientific Reports* **5**: 18206, **doi**: 10.1038/srep18206

Green, T.J., Smullen, R. and Barnes, A.C. (2013) Dietary soybean protein concentrate-induced intestinal disorder in marine farmed Atlantic salmon, *Salmo salar* is associated with alterations in gut microbiota. *Veterinary Microbiology* **166**, 286-292

Guillaume, J. and Choubert, G. (1999) Digestive physiology and nutrient digestibility in fishes. In: *Nutrition and Feeding of Fish and Crustaceans*. (translated by Watson, J.) (Guillaume, G., Kaushik, S., Bergot, P. and Metailler, R. Eds.) pp. 27-58, Springer-Praxis Publishing, Chichester, UK

Hamer, H.M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F.J. and Brummer, R.J. (2008) Review article: the role of butyrate on colonic function. *Alimentary Pharmacology and Therapeutics* **27**, 104-119

Holben, W.E., Williams, P., Gilbert, M., Saarinen, M., Sarkilahti, L.K. and Apajalahti, J.H., (2002) Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. *Microbial Ecology* **44**, 175-185

Ingerslev, H., von Gersdorff Jørgensen, L., Lenz Strube, M., Larsen, N., Dalsgaard, I., Boye, M. and Madsen, L. (2014) The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. *Aquaculture* **424**, 24-34

Jha, R. and Berrocoso, J.F.D. (2016) Dietary fiber and protein fermentation in the intestine of swine and their interactive effects on gut health and on the environment: A review. *Animal Feed Science and Technology* **212**, 18-26

Kaushik, S.J. and Seiliez, I. (2010) Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. *Aquaculture Research* **41**, 322-332

Kim, D.H., Brunt, J. and Austin, B. (2007) Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). *Journal of Applied Microbiology* **102**, 1654-1664

King, G.M., Judd, C., Kuske, C.R. and Smith, C. (2012) Analysis of stomach and gut microbiomes of the eastern oyster (*Crossotrea virginica*) from coastal Louisiana, USA. *PLOS ONE* **7,** e51475

Kormas, K.A., Meziti, A., Mente, E. and Frentzos, A. (2014) Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*). *Microbiology Open* **3**, 718-728

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. and Schloss, P.D. (2013) Development of a dual indexing sequencing strategy and curation pipeline for analyzing

amplicon sequencing data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology* **79**, 5112-5120

Kuz'mina, V.V., Skvortsova, E.G., Shalygin, M.V. and Kovalenko, K.E. (2015) Role of peptidases of the intestinal microflora and prey in temperature adaptations of the digestive system in planktivorous and benthivorous fish. *Fish Physiology and Biochemistry* **41**, 1359-1368

Langille, M.G., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Vega Thurber, R.L., Knight, R., Beiko, R.G. and Huttenhower, C. (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology* **31**, 814-821

Letunic, I., Yamada, T., Kanehisa, M. and Bork, P. (2008) iPath: interactive exploration of biochemical pathways and networks. *Trends in Biochemical Sciences* **33**, 101-103

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H. and Derome, N. (2014) Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in Microbiology* **5**: 207, **doi**: 10.3389/fmicb.2014.00207

Llewellyn, M.S., McGinnity, P., Dionne, M., Letourneau, J., Thonier, F., Carvalho, G.R., Creer, S. and Derome, N. (2015) The biogeography of the Atlantic salmon (*Salmo salar*) gut microbiome. *The ISME Journal* **10**, 1280-1284

Louis, P. and Flint, H.J. (2009) Diversity, metabolism and microbial ecology of butyrate producing bacteria from the human large intestine. *FEMS Microbiology Letters* **294**, 1-8

Lovell, T. (1989) Feed formulation and processing. In: *Nutrition and Feeding of Fish*. pp.175-192, Springer, US

Lovoll, M., Wiik-Nielsen, C.R., Tunsjo, H.S., Colquhoun, D., Lunder, T., Sorum, H. and Grove S. (2009) Atlantic salmon bath challenged with *Moritella viscosa* – pathogen invasion and host response. *Fish and Shellfish Immunology* **26**, 877-884

Lowrey, L., Woodhams, D.C., Tacchi, L. and Salinas, I. (2015) Topographical mapping of the rainbow trout microbiome reveals a diverse bacterial community in the skin with antifungal properties. *Applied and Environmental Microbiology* **81**, 6915-6925

Lozupone, C.A. and Knight, R. (2005) UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* **71**, 8228-8235

Lozupone, C.A. and Knight, R. (2007) Global patterns in bacterial diversity. *Proceedings of the National Academy of Sciences* **104**, 11436-11440

Lunder, T., Evensen, O., Holstad, G. and Halstein, T. (1995) 'Winter ulcer' in the Atlantic salmon *Salmo salar*. Pathological and bacteriological investigations and transmission experiments. *Diseases of Aquatic Organisms* **23**, 39-49

McCune, B., Grace, J.B. and Urban, D.L. (2002) Analysis of ecological communities. MjM Software, Gleneden Beach, Oregon USA

Meadow, N.D., Kukuruzinska, M.A. and Roseman, S. (1985) The bacterial phosphoenolpyruvate: sugar phosphotransferase system. In: *The Enzymes of Biological Membranes* (2nd edn.) (Martonosi, A. Ed.) pp. 523-559, Springer, US

Merrifield, D.L., Balcazar, J.L., Daniels, C., Zhou, Z., Carnevali, O., Sun, Y.Z., Hoseinifar, S.H. and Ringø, E. (2014) Indigenous lactic acid bacteria in fish and crustaceans. In: *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. (Merrifield, D. and Ringø, E., Eds.) pp. 128-168, WILEY Blackwell, Oxford, UK

Miska, K.B., Fetterer, R.H. and Wong, E.A. (2014) The expression of amino acid transporters, aminopeptidase N, and the di- and tri-peptide transporter PepT1 in the embryo of the domesticated chicken (*Gallus gallus*) shows developmental regulation. *Poultry Science* **93**, 2262-2270

Mitrophanov, A.Y. and Groisman, E. (2008) Signal integration in bacterial two-component regulatory systems. *Genes and Development* **22**, 2601-2611

Miyake, S., Ngugi, D.K. and Stingl, U. (2015) Diet strongly influences the gut microbiota of surgeonfishes. *Molecular Ecology* **24**, 656-672

Moran, D., Turner, S.J. and Clements, K.D. (2005) Ontogenetic development of the gastrointestinal microbiota in the marine herbivorous fish *Kyphosus sydneyanus*. *Microbial Ecology* **49**, 590-597

National Research Council (2011) *Nutrient Requirements of Fish and Shrimp*. National Academy Press, Washington D.C.

Nayak, S.K. (2010) Role of gastrointestinal microbiota in fish. *Aquaculture Research* **41,** 1553-1573

Neis, E.P.J.G., Dejong, C.H.C. and Rensen, S.S. (2015) The role of microbial amino acid metabolism in host metabolism. *Nutrients* **7**, 2930-2946

Ozório, R.O.A., Kopecka-Pilarczyk, J., Peixoto, M.J., Lochmann, R., Santos, R.J., Santos, G., Weber, B., Calheiros, J., Ferrez-Arruda, L., Vaz-Pires, P. and Goncalves, J.F.M. (2015) Dietary probiotic supplementation in juvenile rainbow trout (*Oncorhynchus mykiss*) reared under cage culture production: effects on growth, fish welfare, flesh quality and intestinal microbiota. *Aquaculture Research* **doi:** 10.1111/are.12724

Parks, D.H., Tyson, G.W., Hugenholtz, P. and Beiko, R.G. (2014) STAMP: Statistical analysis of taxonomic and functional profiles. *Bioinformatics* **30**, 3123-3124

Pedersen, K., Dalsgaard, I. and Larsen, J.L. (1997) *Vibrio damsela* associated with diseased fish in Denmark. *Applied and Environmental Microbiology* **63**, 711-715

Pourabedin, M. and Zhao, X. (2015) Prebiotics and gut microbiota in chickens. *FEMS Microbiology Letters* **362**, **doi**:10.1093/femsle/fnv122

Rees, D.C., Johnson, E. and Lewinson, O. (2009) ABC transporters: the power to change. *Nature Reviews Molecular Cell Biology* **10**, 218-227

Ringø, E. and Strom, E. (1994) Microflora of Arctic charr, *Salvelinus alpinus* (L): gastrointestinal microflora of free living fish and effect of diet and salinity on intestinal microflora. *Aquaculture and Fish Management* **25**, 623-629

Ringø, E., Strom, E. and Tabachek, J.A. (1995) Intestinal microflora of salmonids: a review. *Aquaculture Research* **26**, 773-789

Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K. and Rawls, J.F. (2011) Evidence for a core gut microbiota in the zebrafish. *The ISME Journal* **5**, 1595-1608

Schloss, P.D. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**, 7537-7541

Schloss, P.D. and Handelsman, J. (2006) Introducing TreeClimber, a test to compare microbial community structures. *Applied and Environmental Microbiology* **72**, 2379-2384

Semova, I., Carten, J.D., Stombaugh, J., Mackey, L.C., Knight, R., Farber, S.A. and Rawls, J.F. (2012) Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. *Cell Host & Microbe* **12**, 277-288

Shaufi, M.A.M, Sieo, C.C., Chong, C.W., Gan, H.M. and Ho, Y.W. (2015) Deciphering chicken gut microbial dynamics based on high throughput 16S r RNA metagenomics analyses. *Gut Pathogens* **7**:4, **doi:** 10.1186/s13099-015-0051-7

Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T., Appel, K.F. and Gram, L. (2000) The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. *Aquaculture* **182**, 1-15

Sugita, H., Isida, Y., Deguchi, Y. And Kadota, H. (1982) Bacterial flora in the gastrointestine of *Tilapia nilotica* adapted in sea water. *Bulletin of the Japanese Society of Scientific Fisheries* **49**, 987-991

Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'Connor, M.P., Rosen, G.L., Knight, R., Kilham, S.S. and Russell, J.A. (2012) Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Molecular Ecology* **21**, 3363-3378

Sullam, K.E., Rubin, B.E., Dalton, C.M., Kilham, S.S., Flecker, A.S., and Russell, J.A. (2015) Divergence across diet, time and populations rules out parallel evolution in the gut microbiomes of Trinadadian guppies. *The ISME Journal* **9,** 1508-1522

Trust, T.J. and Sparrow, R.A.H. (1974) The bacterial flora in the alimentary tract of freshwater salmonid fishes. *Canadian Journal of Microbiology* **20**, 1219-1228

Turnbaugh, P.J. and Gordon, J.I. (2009). The core gut microbiome, energy balance and obesity. *The Journal of Physiology* **587**, 4153-4158.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**, 697-703

White, J.R., Nagarajan, N. and Pop, M. (2009) Statistical methods for detecting differentially abundant features in clinical metagenomics samples. *PLOS Computational Biology* **5**, e1000352

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T., Wiens, G.D., Snekvik, K., Rawls, J.F. and Good, C. (2013) Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. *Applied and Environmental Microbiology* **79**, 4974-4984

Xing, M., Hou, Z., Yuan, J., Liu, Y., Qu, Y. and Liu, B. (2013) Taxonomic and functional metagenomics profiling of gastrointestinal tract microbiome of the farmed adult turbot (*Scopthalmus maximus*) *FEMS Microbiology Ecology* **86**, 432-443

Yamada, T., Letunic, I., Okuda, S., Kanehisa, M. and Bork, P. (2011) iPath 2.0: interactive pathway explorer. *Nucleic Acids Research* **39,** 412-415.

Yoshimizu, M. and Kimura, T. (1976) Studies of the intestinal microflora of salmonids. *Fish Pathology* **10**, 243-259

Yue, J.C. and Clayton, M.K. (2005) A similarity measure based on species proportions. *Communications in Statistics Theory and Methods* **34**, 2123-2131

Zarkasi, K., Abell, G., Taylor, R., Neuman, C., Hatje, E., Tamplin, M., Katouli, M. and Bowman, J.P. (2014) Pyrosequencing based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system. *Journal of Applied Microbiology* **117**, 18-27

Zarkasi, K., Taylor, R.S., Abell, G.C.J, Tamplin, M.L., Glencross, B.D. and Bowman, J.P. (2016) Atlantic Salmon (*Salmo salar* L.) gastrointestinal microbial community dynamics in relation to digesta properties and diet. *Microbial Ecology* **71,** 589-603

4.9. Supplementary information

a)
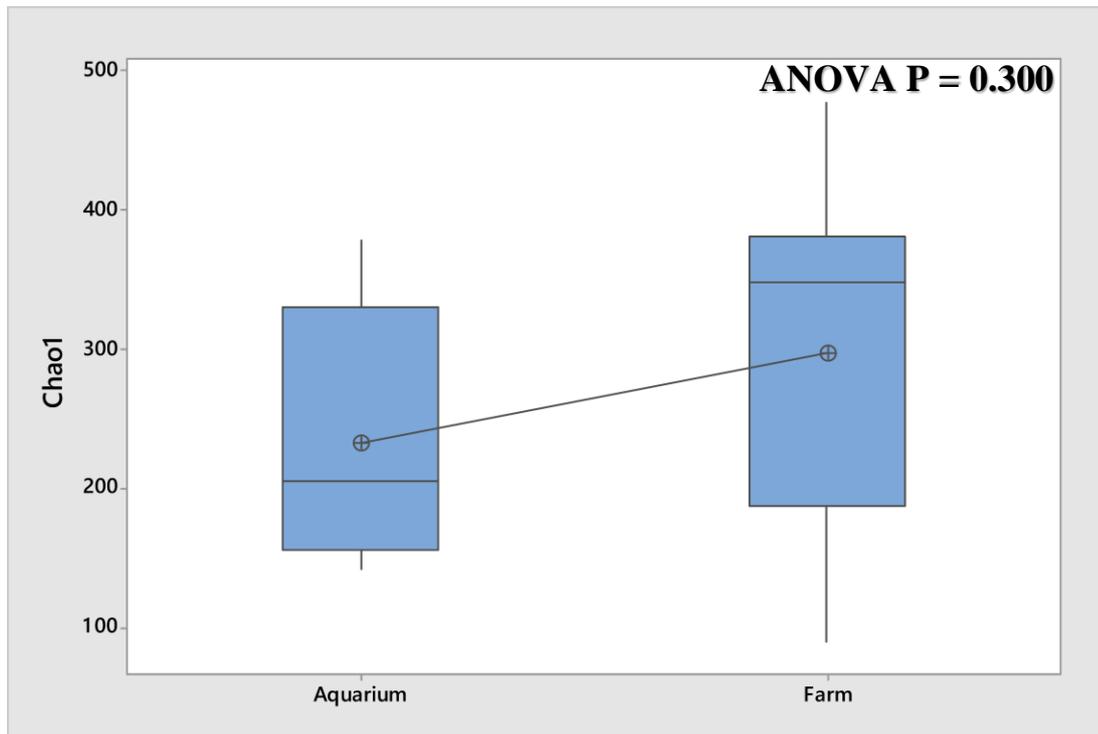
b)
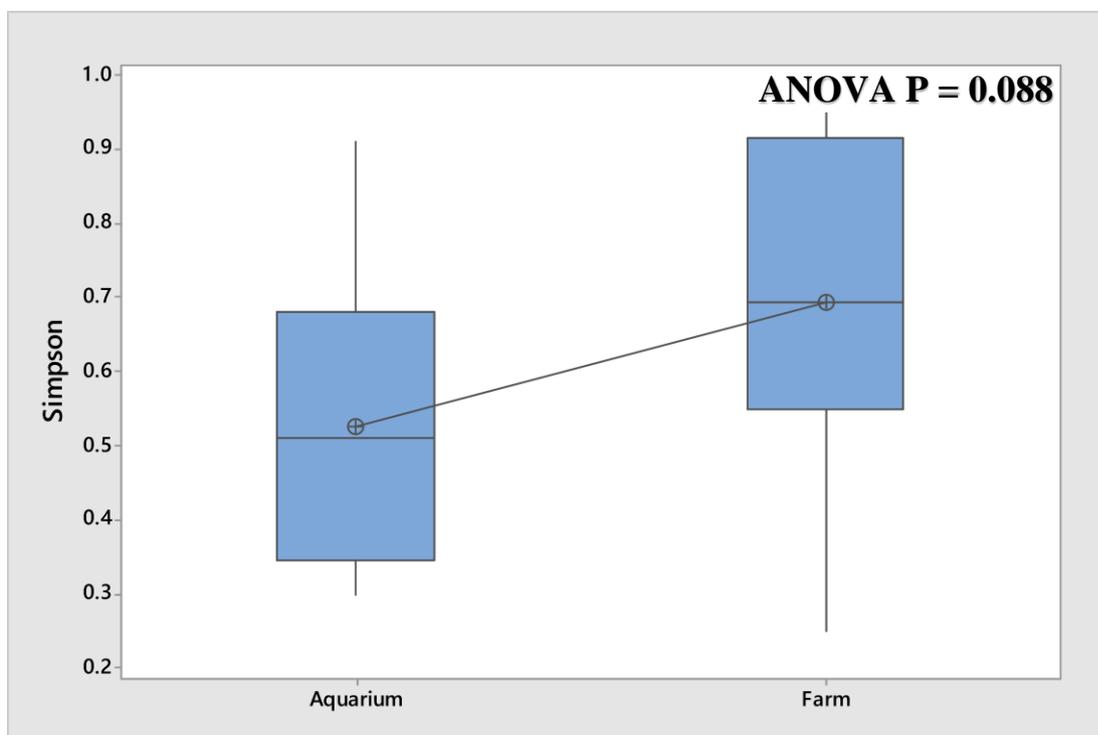
Figure 4.11. (S1) Box plots (median, quartiles and range values) of **a)** Chao1 richness and **b)** Simpson's diversity index of rainbow trout intestinal microbiomes from aquarium (n=9) and farm (n=12).

a)

b)
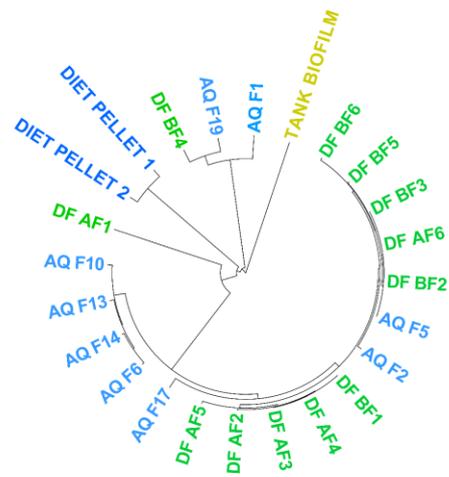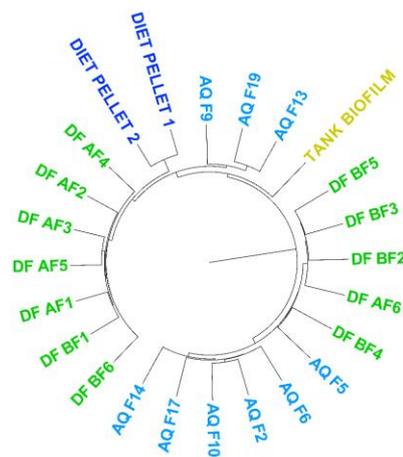
Figure 4.12 (S2) Clustering of samples describing the dissimilarity among tank biofilm, diet pellet, aquarium and farm based rainbow trout intestinal samples according to **a)** ThetaYC and **b)** Jaccard distances.

Chapter 5

General discussion

5.1. Context of this study

The complexity of the predicted functions exerted by the microbiome has led some researchers to refer to it as an 'additional organ' (O'Hara & Shanahan 2006). The available evidence would appear to support this theory in that the bacterial communities that reside in the GI tract of animals play diverse roles in host nutrition, angiogenesis, and the development and maintenance of both the digestive and the immune system (Husebye et al 1994, Stappenbeck et al 2002, Rawls et al. 2004, Gomez & Balcazar 2008, Perez-Sanchez et al 2011). At the outset of this project, however, most of the studies that investigated the intestinal microbiome of fish species had focused on the use of traditional bacterial recovery methods coupled with 'first generation' molecular assays, in determining the identity of the bacteria that comprise this ecosystem (Cahill 1990, Spanggaard et al 2000, Nayak 2010). The difficulties associated with culturing these bacteria, coupled with the restricted resolution of the molecular techniques used, meant that only a fraction of the true microbial diversity had thus far been revealed.

The advent of NGS technologies has permitted more in-depth and comprehensive studies on the composition of the GI microbiota to be performed at a far higher level of resolution. In order to understand the influence of the intestinal microbiome on the health of aquatic animals, it is of the utmost importance that the diversity, structure and function of this microbial ecosystem be unravelled. Recent NGS-based research has begun to improve our appreciation of the complexity of the intestinal microbiome of fish, including rainbow trout, however, there remains a paucity of information regarding the structure of the natural microbiome of healthy animals. The primary aim of this study was therefore to investigate and characterize the distal intestinal microbiome of farmed rainbow trout in order to establish baselines for natural intestinal microbiomes in this species (Chapters 2, 3 and 4). Furthermore, the study aimed to assess the influence of dietary (Chapter 3) and environmental (Chapter 4) factors on the structure of these communities. In addition, the principal functional pathways expressed by the intestinal microbiome of trout from two different rearing environments were explored (Chapter 4) in order to reveal the potential contribution of these microorganisms to host health and nutrition. In short, this thesis aimed to answer three overarching questions: 1) 'Who' is present

in the community? 2) What could they be doing? and 3) What parameters influence these community compositions?

5.2. Summary and conclusions

The elucidation of the true microbial diversity of the fish intestine is an essential first step in developing strategies to fortify and modulate these communities (Al-Hisnawi et al 2014). Early studies in both yellow catfish *Pelteobagrus fulvidraco* and rainbow trout had indicated that the intestinal mucosa harboured a lower level of microbial diversity than that of the lumen (Austin & Al-Zahrani 1988, Spanggaard et al 2000, Kim et al 2007, Wu et al 2010). The aim of the study reported in Chapter 2 was to use high throughput sequencing to investigate the bacterial diversity present in both of these regions within the rainbow trout intestine, in order to present an in-depth overall description of the composition of the microbiome in the fish examined.

The results of this study were in agreement with much of the published literature, in that the major bacterial phyla dominating both regions were the Proteobacteria, Firmicutes and Fusobacteria (Nayak 2010, Romero et al 2014). However, the overall bacterial diversity was greater in the mucosal samples, indicating that this region may be host to a more complex network of communities than previously appreciated. A total of 90 bacterial genera were identified from samples taken from the lumen, compared with 159 from the mucosa. Recent pyrosequencing-based studies of the intestinal microbiome of both snow trout *Schizothorax zarudnyi* (Ghanbari et al 2016) and European sea bass *Dicentrarchus labrax* (Carda-Dieguez et al 2014) have mirrored the findings presented in Chapter 2 in that the composition of the mucosal and luminal microbiota were slightly different in these fish species, with the mucosal region harbouring the greatest diversity.

Altogether, the results of this study suggested that the rainbow trout intestine possesses a very complex microbiome that is more diverse than initial reports had suggested. It is likely that the high throughput sequencing methods employed permitted a greater level of coverage, thus revealing the presence of rare taxa that may remain undetected using less sensitive assays. The composition of the luminal and mucosal communities was slightly different, suggesting that some bacterial phylotypes may be better adapted to each niche. It is accepted that small numbers of fish were examined in the study and so caution must be exercised in comparing these results with other reports of the fish gut microbiome. Furthermore, it has been suggested that the rainbow trout intestinal microbiome can vary according to diet, genotype and rearing

environment (Navarrete et al 2012, Sullam et al 2012, Ringø et al 2015). Nonetheless, this study indicated that high throughput sequencing is a very powerful tool for investigating the microbial diversity of the rainbow trout intestine.

One of the principal aims of this project was to characterize and define bacterial community baselines that are indicative of an apparently healthy intestinal microbiome in rainbow trout, as so little is known about the natural state of this ecosystem in this particular species. Our knowledge on the triggers for an infectious disease outbreak in aquatic farming systems is still being developed and so it is important to understand that fish populations in this study were considered as apparently healthy from key behaviour characteristics combined with lack of clinical signs of disease. The baselines developed in the studies reported here could provide a reference point for future experiments, including screening the effects of novel functional aquafeed ingredients on the intestinal microbiome of fish. It was hypothesized that it may be possible to identify a core group of microbes whose abundance is representative of a balanced microbiome, and furthermore discern the members of the microbiome that may be responsive to modified diets. This hypothesis was tested in Chapter 3, where two groups of rainbow trout were fed slightly different diets over a period of 15 weeks. It was observed that the structure of the intestinal microbiome was similar irrespective of the diets fed, and was dominated by the Tenericutes, Firmicutes and Spirochaetes, indicating that the core microbiome may be resistant to dietary change. However, a subset of the phylum Firmicutes, many of which have previously been identified as receptive to dietary alterations (Ringø et al 2006, Desai et al 2012, Wong et al 2013, Ingerslev et al 2014a, b) were significantly elevated in the treatment group, which could be indicative of their collective ability to metabolize whole-cell microalgal components as an additional fermentable substrate.

These results reflect those of other recent reports in that dietary alterations appear to exert a more pronounced influence over members of the 'auxiliary' microbiome, whereas the core intestinal microbiome remains resistant to such changes (Wong et al 2013). The auxiliary taxa may possibly therefore play important roles in host nutrition, and could represent potential probiotic and prebiotic candidates. *Lactobacillus* and *Streptococcus*, for example, were significantly elevated in the treatment group in Chapter 3, and these genera contain species that are used as probiotic supplements in mammals and fish (Merrifield et al 2010, Ashraf & Shah 2011). The diet dependent differences observed in the structure of the intestinal microbiome raise the possibility that members of the auxiliary microbiome may have contributed to the differences in weight observed at the conclusion of the experiment. Indeed, Wong et al (2013)

observed a similar trend involving many of the same taxa identified as discriminatory by diet in Chapter 3. However, the differences in fish weight recorded in Chapter 3 were not statistically significant, and so further research is required to explore this hypothesis.

Furthermore, the findings from Chapter 3 illustrate the advantage of high throughput sequencing in detecting diet associated perturbations of the intestinal microbiome. The results also emphasized the importance of characterizing the intestinal microbiome of individual fish rather than pooled samples, as substantial variation was observed between individuals in this study. Taken together, these data represent detailed microbiome baselines in healthy fish, and suggest that it may be possible to shape the composition of the rainbow trout intestinal microbiome with bespoke feeding strategies that target receptive members of the bacterial community, whose active role in gut physiology may influence fish health (Tellez et al 2006, Daniels & Hoseinifar 2014, Llewellyn et al 2014, Ringø et al 2014). This could have important implications for the aquafeed industry, particularly at a time when new feed sources and raw materials are being sought to alleviate the environmental footprint of aquaculture.

Previous studies have shown that the intestinal microbiome of farmed fish can display significant variation according to the geographical location of their rearing environment (Ringø et al 1995, Holben et al 2002, Sullam et al 2012, Giatsis et al 2015). Therefore, the study described in Chapter 4 aimed to assess whether the baseline microbiomes observed in aquarium based fish were comparable to those of fish raised in a freshwater commercial cage system. Furthermore, in this chapter, a novel metagenomics tool, PICRUSt, was applied in order to investigate the principal functional pathways expressed by the intestinal microbiome of rainbow trout.

The results showed that the intestinal microbiome of both of the populations of fish tested were similar irrespective of the differences in rearing environment. This suggests that the structure of the rainbow trout intestinal microbiome may be robust to changes in farming environment and could point to a more complex level of interdependence with the host. A significant difference was, however, noted in community membership which suggests that the farming environment exerts at least some influence on the intestinal microbiome, possibly by acting as a source of colonizing microbes that are distinct to each geographical location. This assertion is supported by the elevated abundance of more scarce psychrophilic bacterial taxa in the farm based samples, and may possibly be explained by the unseasonally low temperatures recorded at the time of sampling. It is therefore likely that a combination of determininstic host effects

and stochastic environmental factors underpin bacterial diversity in the rainbow trout intestinal microbiome, a pattern that has recently been noted in Atlantic salmon (Llewellyn et al 2015), and these data indicate that host selection within the intestinal tract may be more influential in shaping the overall community structure. Similarities in the gut microbiomes of individual fish species sampled from different localities have also previously been observed in halibut *Hippoglossus hippoglossus* (Jensen et al 2004), zebrafish *Danio rerio* (Roeselers et al 2011, Yan et al 2012) and cod *Gadhus morhua* (Bakke et al 2015) and so the data collected in this chapter suggest that a similar deterministic pattern of colonization may also occur in farmed rainbow trout.

PICRUSt analysis was applied to the 16S rRNA libraries of the fish sampled in Chapter 4 in order to focus on the predicted microbial activity within the intestinal tract, thereby providing a more robust description of the intestinal microbiome and its potential influence on the physiology of rainbow trout. This approach has been successfully applied in other animals (Corrigan et al 2015, Shaufi et al 2015), but at the time of writing only a very limited number of studies have utilized this approach in farmed fish and shellfish species (Cleary et al 2015, Wu et al 2015, Liu et al 2016). The majority of functional pathways expressed in the Chapter 4 libraries contained genes responsible for metabolism, transport and cellular processes. Overall, it was observed that protein metabolism pathways were dominant, a trend that may be linked to the high protein nature of salmonid aquafeed formulations. Indeed, in terrestrial farm animals whose diets contain a high level of carbohydrate, functional metagenomic analyses of the GI microbiome have shown that pathways relating to carbohydrate metabolism are the most dominant (Corrigan et al 2015, Pourabedin & Zhao 2015).

It is known that the distal intestine of rainbow trout is a primary site for the uptake of protein in teleost fish (Guillaume & Choubert 1999), and so the enhanced KEGG pathways attributed to peptidase production and amino acid synthesis suggest that the microbiome may contribute to the improvement of this process in this region. This may be of particular importance in temperate species such as rainbow trout, whose endogenous pancreatic enzyme activity decreases in lower water temperatures (Kuz'mina et al 2015). Microorganisms that synthesize peptidases and other extracellular enzymes have been shown to hydrolyze the same substrates as the enzymes synthesized by the fish digestive tract, and this has been demonstrated for many of the bacterial phylotypes observed in Chapter 4 (Skrodenyte-Arbaciaskiene 2000, Ghosh et al 2002, Esakkiraj et al 2009, Ray et al 2010, Askarian et al 2012). Therefore, the data collected in Chapter 4 demonstrate that the activity of the intestinal microbiome may compensate for and

reinforce the action of endogenous digestive enzymes in the rainbow trout intestine. Conversely, pathways attributed to carbohydrate metabolism, whilst present, were not as prevalent in the present study, possibly owing to the low carbohydrate content of rainbow trout feed in comparison to the diets of terrestrial farm animals.

In summary, the data presented in Chapter 4: 1) further represent baselines for healthy rainbow trout intestinal microbiomes, 2) suggest that the composition of these baselines is driven primarily by deterministic factors, and 3) demonstrate that the microbiome may be actively involved in the metabolism of dietary ingredients, and may therefore contribute to the digestive physiology of rainbow trout. However, future studies should focus on measuring bacterial metabolites in the intestine of these fish in order to subsequently quantify the extent of this contribution, and to complement and support the findings from the PICRUSt pipeline. Furthermore, it should be noted that this approach is inherently limited to those gene pathways that have been previously described in the literature and subsequently deposited in gene databases. Thus, further work is required to continue to annotate functions to poorly characterized bacterial gene pathways in order to fully realize the contribution made by the intestinal microbiome to rainbow trout nutrition and health. Nonetheless, through identifying the metagenomic capacity of the rainbow trout intestinal microbiome, it may be possible to develop future feeding strategies to enrich those pathways that actively influence host digestion, thus harnessing the metabolic potential of these microorganisms and consequently improving fish health and productivity.

5.3. Further comments and future work

There is a discrepancy between the composition of the intestinal microbiome as described in Chapter 2 when compared with that of Chapter 3 and 4. Moreover, further work, not included in this thesis but being prepared for publication, produced similar microbiome compositions to those reported in Chapter 3 and 4. The exact cause of this discrepancy is unclear, however a number of possibilities may have contributed. Firstly, an anti-fungal treatment had been administered to these fish two weeks prior to sampling. Therefore, it is possible that the microbiome may not have returned to its baseline composition after this disturbance. Moreover, the use of chemical treatments to control or prevent specific pathogens has previously been shown to alter the composition of the fish microbiome (Boutin et al 2013, Mohammed & Arias 2015, Narrowe et al 2015, Gaulke et al 2016). Secondly, these fish were fed a different diet to those sampled in the other experiments described in this thesis and were maintained in a lower

stocking density than the other fish sampled. Both diet and rearing density have been shown to influence the structure and composition of the intestinal microbiome of a range of fish species to varying degrees (Zhou et al 2011, Carda-Dieguez et al 2014, Wong et al 2013, Givens 2014, Kormas et al 2014, Larsen et al 2014, Miyake et al 2015). Finally, in Chapter 2 the samples were held on ice before transfer to the laboratory, whereas in Chapter 3 and 4 dry ice was used. Any one of these factors may have contributed to the differences observed in the community composition of the fish sampled in Chapter 2. It should be noted however that previous NGS-based studies of the rainbow trout microbiome have also reported intestinal community compositions similar to both Chapter 2 (Desai et al 2012, Wong et al 2013, Ingerslev et al 2014a, b), and Chapter 3 and 4 (Holben et al 2002, Zarkasi et al 2014, Lowrey et al 2015, Llewellyn et al 2015, Etyemez & Balcazar 2015), indicating that further research is required to explore the trends reported in this thesis, employing some of the following recommendations.

To better understand the composition of the intestinal microbiome, it would be beneficial to employ high throughput sequencing techniques to characterize these microorganisms at all life stages, from egg to adult. This would permit direct comparisons between studies and allow researchers to quantify how dynamic these communities are within the rainbow trout intestine, throughout each phase of their life cycle. Ideally, it would be advantageous to profile the intestinal microbiome of individual fish at the beginning of a trial, and to then monitor those fish at multiple stages throughout the duration of the experiment. This would enhance our understanding of how labile the intestinal microbiome is over time and enable an improved observation of the response of the communities to different dietary or environmental factors. This approach is not currently possible for trout, without firstly anaesthetizing and then reviving the fish once a sample has been collected, usually via the insertion of a catheter through the vent. The response of such fish and their intestinal microbiomes to the potential stress associated with repeated anaesthesia and revival over the time course of such a trial is unknown. However, recent research has shown encouraging signs that it may be possible to monitor the succession and dynamics of the intestinal microbiome of zebrafish using a novel light sheet microscopy approach which can produce live images of tagged bacterial communities in the gut over time (Stephens et al 2015). Although the morphology of zebrafish makes them ideal candidates for such a method, it is possible that similar tools may be developed in the future for the non-invasive real-time analysis of the gut microbiome of rainbow trout and other commercially important aquaculture species.

Furthermore, novel and innovative methods are currently being successfully applied to improve the level of recovery of intestinal bacteria from the human GI tract (Goodman et al 2011, Lagier et al 2012, Browne et al 2016). The future implementation of these techniques in the study of the fish gut microbiome will unlock the phenotypic characteristics of many fastidious bacteria, previously considered 'unculturable', and consequently improve our knowledge of the functional potential of these communities. Once cultured, it may also be possible to develop in-vitro fermentation models of the fish gut in order to examine the community dynamics of the microbiome, and its responses to experimental variations, without the need to sacrifice the animal. Such models have been successfully developed in terrestrial farmed animals as a tool to monitor feed efficiency (Cardozo et al 2004, Patra & Yu 2012, 2013).

Early studies suggested that the structure of the intestinal microbiome of rainbow trout is highly variable according to each region of the intestine, however more recent research has shown that the composition may be similar in both the anterior and distal regions (Lowrey et al 2015). The distal intestine was chosen in the present research for three main reasons: 1) this region has been reported to harbour the greatest level of bacterial diversity in salmonid fish (Ringø et al 1995, Nayak 2010), 2) the proximity of this region in relation to the principal sites of endogenous gastric and pancreatic enzyme production means that these factors are less likely to exert a strong influence on the community composition, and 3) hindgut bacterial fermentation has been reported in other fish species (Mountfort et al 2002, Clements et al 2014). However, it would be advantageous for future studies to employ high-throughput sequencing technologies to examine the microbiome in the different regions of the GI tract of rainbow trout in order to describe the relative contribution of the microbiome in each of these regions to the digestive physiology of these fish.

Further improvements could be made in study design. In Chapter 3, spatial and financial constraints meant that a limited number of individual fish were examined over a defined period of time. However, through applying the same study design to a larger number of fish, and perhaps to multiple cohorts, much greater statistical power could be achieved and hence may elucidate some of the trends observed in this work. This may be particularly important in studies assessing microbial variation within populations as microbiome studies are often faced with many potential confounding factors such as host lifestyle, disease and genetics, which can also play important roles in shaping the microbial community. Such an analysis involving large numbers of individual fish will most likely be possible in the near future as the cost of high throughput sequencing technologies continues to fall.

Whilst many of the more recent studies have concentrated on profiling the taxonomic composition of the fish gut microbiome, it is likely that future research will continue to build on the experimental design of Chapter 4 in exploring the activity of these microorganisms in greater detail. There are currently very few published studies available that have investigated the link between the structure of the intestinal microbiome and its function in fish (Xing et al 2013, Xia et al 2014, Ghanbari et al 2015, Wu et al 2015, Liu et al 2016). To the author's knowledge, the study reported in Chapter 4 is the first to investigate this relationship in rainbow trout, and this chapter provides novel insights into the potential functional contribution of the microbiome in this fish species. Metagenomic analyses hold a lot of promise for future microbiome research in that they permit the entire genetic complement of a microbial community to be interrogated and can be used in tandem with metatranscriptomic, metaproteomic and metabolomic assays, in a high-throughput approach. Metatranscriptomics involves the retrieval and sequencing of mRNA transcipts from a complex environment, such as the fish GI tract, and determining the active bacterial taxa in that environment by assessing which genes are being expressed. Such an approach has been applied to explore the activity of members of the human gut microbiome after dietary alterations (McNulty et al 2011), and could reveal important trends in the fish intestine. This may be particularly pertinent given the environmental challenges that the aquaculture industry is currently facing regarding the sustainable sourcing of novel aquafeed ingredients and their as yet unknown effects on fish health and growth.

Furthermore, metaproteomic methods can be used to identify the microbial proteins translated in a complex sample by matching their sequences with the available databases. In this way, putative functions can be assigned that reflect the functional potential of the microbiota in the original sample (Sorek & Cossart 2010, Franzosa et al 2015). Finally, metabolomics refers to the detection of bacterial metabolites and other small molecules in microbial communities. Thus far only a single study has combined high-throughput metagenomic and metabolomic approaches to profile the composition of the microbiome and its metabolites in the fish gut (Asakura et al 2014), and these authors demonstrated the potential to manipulate microbial metabolites through different feeding strategies. By identifying the microbial metabolites produced in the rainbow trout intestine, future work will similarly determine the contribution of these metabolites to host nutrition and health at both local and systemic levels. The challenge for future research will be to combine these complementary meta-omics approaches with other novel techniques, so as to formulate an ecosystem-level strategy to study the structure and

function of the intestinal microbiome of fish (Fig. 5.1). Such a strategy will undoubtedly provide a more robust understanding of the interaction between the intestinal microbiome and important aquaculture species, and unravel the totality of the influence of this 'additional organ' on fish physiology and health.
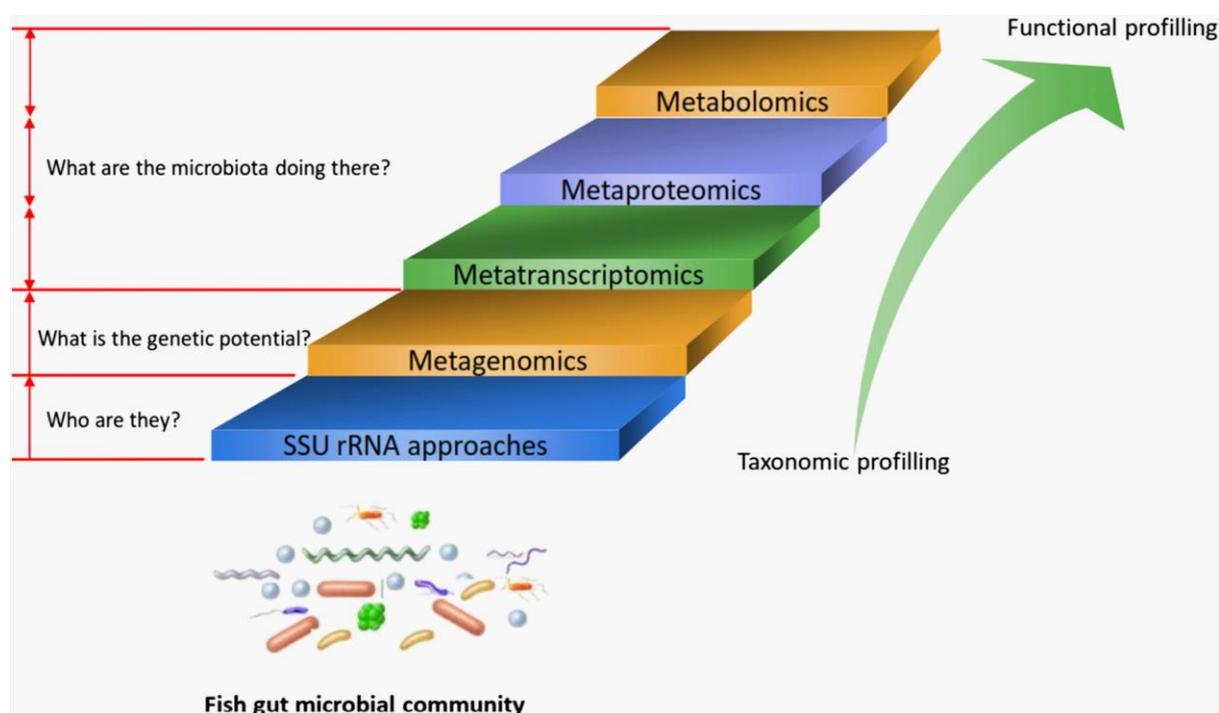

Figure 5.1. Simplified model describing the meta-omic approach which will permit researchers to elucidate the structure and function of microbiome samples. Metagenomic approaches should be complemented by the parallel detection of expressed mRNA transcripts (metatranscriptomics), translated proteins (metaproteomics) and the metabolites produced (metabolomics). Source: Ghanbari et al (2015).

In conclusion, the data in Chapter 2 showed that the microbial diversity of the distal intestine of rainbow trout is much more complex and diverse than previously appreciated. This chapter also demonstrated that the lumen and mucosa may host different microbial communities and illustrated the power of high throughput sequencing approaches in determining the structure of the intestinal microbiome. Chapter 3 investigated the effects of dietary alteration on the structure of the distal intestinal microbiome in rainbow trout. The results indicated that dietary supplementation with ALL-G-Rich™ microalgae did not alter overall microbial community structure, but caused shifts in specific LAB taxa, many of which are viewed as beneficial organisms within the salmonid intestine. Chapter 4 revealed that the structure of the intestinal microbiome of rainbow trout based in both aquarium and farm settings was not significantly

different despite the differences in rearing environment. Furthermore, this chapter is one of the first studies to have revealed insights into the predicted functional potential of the intestinal microbiome of rainbow trout, and the data indicate a potentially important role for these communities in the digestive physiology of this species.

The work presented in this thesis contributes to a growing body of knowledge on the structure of the intestinal microbiome in commercially important cultured fish species. The ever increasing demand for seafood, driven by a burgeoning human population, has precipitated a need for more sustainable aquafeed ingredients that lessen the burden on the aquatic environment whilst improving the health of farmed fish and the productivity of fish farmers. It is therefore imperative that these feed ingredients, such as the microalgae meal used in this thesis, be tested in rainbow trout and other key aquaculture species worldwide, using the full complement of novel molecular tools now available. Such research will continue to enhance our understanding of how the functional potential of the microbiome can be harnessed to improve fish health, and consequently the health of the human consumer.

5.4. References

Al-Hisnawi, A., Ringø, E., Davies, S.J., Waines, P., Bradley, G. and Merrifield, D.L. (2014) First report on the autochthonous gut microbiota of brown trout (*Salmo trutta* Linnaeus). *Aquaculture Research* **46**, 2962-2971

Asakura, T., Sakata, K., Yoshida, S., Date, Y. and Kikuchi, J. (2014) Noninvasive analysis of metabolic changes following nutrient input into diverse fish species, as investigated by metabolic and microbial profiling approaches. *PeerJ* **2**: e550.10.7717/peerj.550

Ashraf, R. and Shah, N.P. (2011) Selective and differential enumerations of *Lactobacillus delbrueckii* subs. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt − a review. *International Journal of Food Microbiology* **149**, 194-208

Askarian, F., Zhou, Z., Olsen, R.E., Sperstad, S. and Ringø, E. (2012) Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and in vitro growth inhibition of four fish pathogens. *Aquaculture* **326**, 1-8

Austin, B. and Al-Zahrani, A.M.J. (1988). The effect of antimicrobial compounds on the gastrointestinal microflora of rainbow trout *Salmo gairdneri* Richardson. *Journal of Fish Biology* **33**, 1-14

Bakke, I., Coward, E., Andersen, T. and Vadstein, O. (2015). Selection in the host structures the microbiota associated with developing cod larvae (*Gadhus morhua*). *Environmental Microbiology* **17**, 3914-3924

Boutin, S., Bernatchez, L., Audet, C. and Derôme, N. (2013) Network analysis highlights complex interactions between pathogen, host and commensal microbiota. *PLOS ONE* **8**, e84772

Browne, H.P., Forster, S.C., Anonye, B.O., Kumar, N., Neville, B.A., Stares, M., Goulding, D. and Lawley, T.D. (2016) Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature* **533,** 543-546

Cahill, M.M. (1990) Bacterial flora of fishes: A review. *Microbial Ecology* **19**, 21-41

Carda-Dieguez, M., Mira, A. and Fouz, B. (2014) Pyrosequencing survey of intestinal microbiota diversity in cultured sea bass (*Dicentrarchus labrax*) fed functional diets. *FEMS Microbiology Ecology* **87**, 451-459

Cardozo, P.W., Calsamiglia, S., Ferret, A. and Kamel, C. (2004) Screening for the effects of natural plant extracts at different p H on in vitro rumen microbial fermentation of a high-concentrate diet from beef cattle. *Journal of Animal Science* **83**, 2572-2579

Cleary, D.F., Becking, L.E., Polonia, A.R.M., Freitas, R.M. and Gomes, N.C.M. (2015) Composition and predicted functional ecology of mussel-associated bacteria in Indonesian marine lakes. *Antonie van Leeuwenhoek* **107**, 821-834

Clements, K.D., Angert, E.R., Montgomery, W.L. and Choat, J.H. (2014) Intestinal microbiota in fishes: what's known and what's not. *Molecular Ecology* **23**, 1891-1898

Corrigan, A., De Leeuw, M., Penaud-Frezet, S., Dimova, D. and Murphy, R.A. (2015) Phylogenetic and functional alterations in bacterial community compositions in broiler ceca as a result of mannan oligosaccharide supplementation. *Applied and Environmental Microbiology* **81**, 3460-3470

Daniels, C. and Hoseinifar, S.H. (2014) Prebiotic applications in shellfish. In: *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. (1st edn.) (Merrifield, D. and Ringø, E. Eds.) pp. 401-419. WILEY Blackwell, Oxford, UK.

Desai, A.R., Links, M.G., Collins, S.A., Mansfield, G.S., Drew, M.D., Van Kessel, A.G. and Hill, J.E. (2012) Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **350**, 134-142

Esakirraj, P., Immanuel, G., Sowmya, S.V., Iyapparaj, P. and Palavesam, A. (2009) Evaluation of protease-producing ability of fish gut isolate *Bacillus cereus*. *Food Bioprocess Technology* **2**, 383-390

Etyemez, M. and Balcazar, J.L. (2015) Bacterial community structure in the intestinal ecosystem of rainbow trout (*Oncorhynchus mykiss*) as revealed by pyrosequencing-based analysis of 16S rRNA genes. *Research in Veterinary Science* **100**, 8-11

Franzosa, E.A., Hsu, T., Sirotas-Madi, A., Shafquat, A., Abu-Ali, G., Morgan, X.C. and Huttenhower, C. (2015) Sequencing and beyond: integrating molecular 'omics' for microbial community profiling. *Nature Reviews Microbiology* **13,** 360-372

Gaulke, C.A., Barton, C.L., Proffitt, S., Tanguay, R.L. and Sharpton, T.J. (2016) Triclosan exposure is associated with rapid restructuring of the microbiome in adult zebrafish. *PLOS ONE* **11**, e0154632

Ghanbari, M., Kneifel, W. and Domig, K. (2015) A new view of the fish gut microbiome: Advances from next-generation sequencing. *Aquaculture* **448,** 464-475

Ghanbari, M., Shahraki, H., Kneifel, W. and Domig, K.J. (2016) Community membership and structure of mucosa and luminal-associated intestinal microbiota in snow trout (*Schizothorax zarudnyi*) as revealed by pyrosequencing. *FEMS Microbiology Ecology* (in press)

Ghosh, K., Sen, S.K. and Ray, A.K. (2002) Characterization of bacilli isolated from the gut of rohu, *Labelio rohita*, fingerlings and its significance in digestion. *Journal of Applied Aquaculture* **12**, 33-42

Giatsis, C., Sipkema, D., Smidt, H., Heilig, H., Benvenuti, G., Verreth, J. and Verdegem, M. (2015) The impact of rearing environment on the development of gut microbiota in tilapia larvae. *Scientific Reports* **5**: 18206, **doi:** 10.1038/srep18206

Givens, C. (2014) A fish tale: comparison of the gut microbiome of 15 fish species and the influence of diet and temperature on its composition. Department of Marine Sciences, University of Georgia, Georgia, USA, Doctoral Thesis, pp. 218-221. Available online at: https://getd.libs.uga.edu/pdfs/givens_carrie_e_201212_phd.pdf

Gomez, G.D. and Balcazar, J.L. (2008) A review on the interactions between gut microbiota and innate immunity of fish. *FEMS Immunology and Medical Microbiology* **52**, 145-154

Goodman, A.L., Kallstrom, G., Faith, J.J., Reyes, A., Moore, A., Dantas, G. and Gordon, J.I. (2011) Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. *Proceedings of the National Academy of Sciences of the USA* **108**, 6252-6257

Guillaume, J. and Choubert, G. (1999) Ontogeny, development and digestive physiology of fish larvae. In: *Nutrition and Feeding of Fish and Crustaceans*. (translated by Watson, J.) (Guillaume, G., Kaushik, S., Bergot, P. and Metailler, R. Eds.) pp. 199-212, Springer-Praxis Publishing, UK

Holben, W.E., Williams, P., Gilbert, M., Saarinen, M., Sarkilahti, L.K. and Apajalahti, J.H., (2002) Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. *Microbial Ecology* **44**, 175-185

Husebye, E., Hellström, P.M. and Midtvedt, T. (1994) Intestinal microflora stimulates myoelectric activity of rat small intestine by promoting cyclic initiation and aboral propagation of migrating myoelectric complex. *Digestive Diseases and Sciences* **39**, 946-956

Ingerslev, H.C, von Gersdorff Jørgensen, L., Lenz Strube, M., Larsen, N., Dalsgaard, I., Boye, M. and Madsen, L. (2014a) The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. *Aquaculture* **424**, 24-34

Ingerslev, H.C., Strube, M.L., Jørgensen, L., Dalsgaard, I., Boye, M. and Madsen, L. (2014b) Diet type dictates the gut microbiota and the immune response against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology* **40**, 624-633

Jensen, S., Ovreas, L., Bergh, Ø. and Torsvik, V. (2004) Phylogenetic analysis of bacterial communities associated with larvae of the Atlantic halibut propose succession from a uniform normal flora. *Systematic Applied Microbiology* **27**, 728-736

Kim, D.H., Brunt, J. and Austin, B. (2007) Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). *Journal of Applied Microbiology* **102**, 1654-1664

Kormas, K.A., Meziti, A., Mente, E. and Frentzos, A. (2014) Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*). *Microbiology Open* **3**, 718-728

Kuz'mina, V.V., Skvortsova, E.G., Shalygin, M.V. and Kovalenko, K.E. (2015) Role of peptidases of the intestinal microflora and prey in temperature adaptations of the digestive system in planktivorous and benthivorous fish. *Fish Physiology and Biochemistry* **41**, 1359-1368

Lagier, J.C., Armougom, F., Million, M., Hugon, P., Pagnier, I., Robert, C., Bittar, F., Fournous, G., Gimenez, G., Maraninchi, M., Trape, J.F., Koonin, E.V., La Scola, B. and Raoult, D. (2012) Microbial culturomics: paradigm shift in the human gut microbiome study. *Clinical Microbiology and Infection* **18**, 1185-1193

Larsen, A.M., Mohammed, H.H. and Arias, C.R. (2014) Characterization of the gut microbiota of three commercially valuable warm water species. *Journal of Applied Microbiology* **116**, 1396-1404

Liu, H., Guo, X., Gooneratne, R., Lai, R., Zeng, C., Zhan, F. and Wang, W. (2016) The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels. *Scientific Reports* **6:**24340 doi: 10.1038/srep243340

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H. and Derome, N. (2014) Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in Microbiology* **5**:207, **doi:** 10.3389/fmicb.2014.00207

Llewellyn, M.S., McGinnity, P., Dionne, M., Letourneau, J., Thonier, F., Carvalho, G.R., Creer, S. and Derome, N. (2015) The biogeography of the Atlantic salmon (*Salmo salar*) gut microbiome. *The ISME Journal* **10**, 1280-1284

Lowrey, L., Woodhams, D.C., Tacchi, L. and Salinas, I. (2015) Topographical mapping of the rainbow trout microbiome reveals a diverse bacterial community in the skin with antifungal properties. *Applied and Environmental Microbiology* **81**, 6915-6925

McNulty, N.P., Yatsunenko, T., Hsiao, A., Faith, J.J., Muegge, B.D., Goodman, A.L., Henrissat, B., Oozeer, R., Cools-Portier, S., Gobert, G., Chervaux, C., Knights, D., Lozupone, C.A., Knight, R., Duncan, A.E., Bain, J.R., Muehlbauer, M.J., Newgard, C.B., Health, A.C. and Gordon, J.I. (2011) The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. *Science Translational Medicine* **3, doi:** 10.1126/scitranslmed.3002701

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bøgwald, J., Castex, M. and Ringø, E. (2010) The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* **302**, 1-18

Miyake, S., Ngugi, D.K. and Stingl, U. (2015) Diet strongly influences the gut microbiota of surgeonfishes. *Molecular Ecology* **24**, 656-672

Mohammed, H.H. and Arias, C.R. (2015) Potassium permanganate elicits a shift of the external fish microbiome and increases host susceptibility to columnaris disease. *Veterinary Research* **46**:82, **doi**: 10.1186/s13567-015-0215-y

Mountfort, D.O., Campbell, J. and Clements, K.D. (2002) Hindgut fermentation in three species of marine herbivorous fish. *Applied and Environmental Microbiology* **68**, 1374-1380

Narrowe, A.B., Albuthi-Lantz, M., Smith, E.P., Bower, K.J., Roane, T.M., Vajda, A.M. and Miller, C.S. (2015) Perturbation and restoration of the fathead minnow gut microbiome after low-level triclosan exposure. *Microbiome* **3**:6, **doi**: 10.1186/s40168-015-0069-6

Navarrete, P., Magne, F., Araneda, C., Fuentes, P., Barros, L., Opazo, R., Espejo, R. and Romero, J. (2012) PCR-TTGE analysis of 16S rRNA from rainbow trout (*Oncorhynchus mykiss*) gut microbiota reveals host-specific communities of active bacteria. *PLOS ONE* **7**, e31335

Nayak, S.K. (2010) Role of gastrointestinal microbiota in fish. *Aquaculture Research* **41,** 1553-1573

O'Hara, A. and Shanahan, F. (2006) The gut flora as a forgotten organ. *EMBO Reports* **7**, 688-693

Patra, A.K. and Yu, Z. (2012) Effects of essential oils on methane production and fermentation by, and abundance and diversity of, rumen microbial populations. *Applied and Environmental Microbiology* **78**, 4271-4280

Patra, A.K. and Yu, Z. (2013) Effects of coconut and fish oils on ruminal methanogenesis, fermentation, and abundance and diversity of microbial populations in vitro. *Journal of Dairy Science* **96**, 1782-1792

Perez-Sanchez, T., Balcazar, J.L., Merrifield, D.L., Carnevali, O., Gioacchini, G., de Blas, I. and Ruiz-Zarzuela, I. (2011) Expression of immune-related genes in rainbow trout (*Oncorhynchus mykiss*) induced by probiotic bacteria during *Lactococcus garviae* infection. *Fish and Shellfish Immunology* **31**, 196-201

Pourabedin, M. and Zhao, X. (2015) Prebiotics and gut microbiota in chickens. *FEMS Microbiology Letters* **362, doi**: 10.1093/femsle/fnv122

Rawls, J.F., Samuel, B.S. and Gordon, J.I. (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the USA* **101**, 4596-4601

Ray, A.K., Roy, T., Mondal, S. and Ringø, E. (2010) Identification of gut-associated amylase, cellulase and protease-producing bacteria in three species of Indian major carps. *Aquaculture Research* **41**, 1462-1469

Ringø, E., Strom, E. and Tabachek, J.A. (1995) Intestinal microflora of salmonids: a review. *Aquaculture Research* **26**, 773-789

Ringø, E., Sperstad, S., Myklebust, R., Mayhew, T.M. and Olsen, R.E. (2006) The effect of dietary inulin on bacteria associated with the hindgut of Arctic charr (*Salvelinus alpinus* L.). *Aquaculture Research* **37**, 891-897

Ringø, E., Dimitroglou, A., Hoseinifar, S.H. and Davies, S.J. (2014) Prebiotics in finish: an update. In: *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. (1st edn) (Merrifield, D. and Ringø, E. Eds.) pp. 360-401, WILEY Blackwell, Oxford, UK.

Ringø, E., Zhou, Z., Vecino, J.L.G., Wadsworth, S., Romero, J., Krogdahl, Å., Olsen, R.E., Dimitroglou, A., Foey, A., Davies, S., Owen, M., Lauzon, H.L., Martinsen, L.L., De Schryver, P., Bossier, P., Sperstad, S. and Merrifield, D. (2015) Effect of dietary components on the gut microbiota of aquatic animals. A never ending story? *Aquaculture Nutrition* **22**, 219-282

Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K. and Rawls, J.F. (2011) Evidence for a core gut microbiota in the zebrafish. *The ISME Journal* **5**, 1595-1608

Romero, J., Ringø, E. and Merrifield, D. (2014) The gut microbiota of fish. In: *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. (Merrifield, D. and Ringø, E., Eds.) pp. 75-100, WILEY Blackwell, Oxford, UK.

Shaufi, M.A.M, Sieo, C.C., Chong, C.W., Gan, H.M. and Ho, Y.W. (2015) Deciphering chicken gut microbial dynamics based on high throughput 16S r RNA metagenomics analyses. *Gut Pathogens* **7**:4, **doi:** 10.1186/s13099-015-0051-7

Skrodenyte-Arbaciaskiene, V. (2000) Proteolytic activity of the roach (*Rutilus rutilus*) intestinal microflora. *Acta Zoologica Lituanica* **10**, 69-77

Sorek, R. and Cossart, P. (2010) Prokaryotic transcriptomics: a new view on regulation, physiology and pathogenicity. *Nature Reviews Genetics* **11,** 9-16

Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T., Appel, K.F. and Gram, L. (2000) The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. *Aquaculture* **182**, 1-15

Stappenbeck, T., Hooper, L.V. and Gordon, J. (2002) Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proceedings of the National Academy of Sciences of the USA* **99**, 15451-15455

Stephens, W.Z., Wiles, T.W., Martinez, E.S., Jemielita, M., Burns, A.R., Parthasarathy, R., Bohannan, B.J.M. and Guillemin, K. (2015) Identification of population bottlenecks and colonization factors during assembly of bacterial communities within the zebrafish intestine. *mBio* **6**: e01163-15, **doi**: 10.1128/mBio.01163-15

Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'Connor, M.P., Rosen, G.L., Knight, R., Kilham, S.S. and Russell, J.A. (2012) Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Molecular Ecology* **21**, 3363-3378

Tellez, G., Higgins, S., Donaghue, A. and Hargis, B. (2006) Digestive physiology and the role of microorganisms. *Journal of Applied Poultry Research* **15,** 136-144

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T., Wiens, G.D., Snekvik, K., Rawls, J.F. and Good, C. (2013). Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. *Applied and Environmental Microbiology* **79**, 4974-4984

Wu, S., Gao, T., Zheng, Y., Wang, W., Cheng, Y. and Wang, G. (2010) Microbial diversity of intestinal contents and mucus in yellow catfish (*Pelteobagrus fulvidraco*). *Aquaculture* **303**, 1-7

Wu, S., Ren, Y., Peng, C., Hao, Y., Xiong, F., Wang, G., Wenxiang, L., Zou, H. and Angert, R.R. (2015) Metatranscriptomic discovery of plant biomass-degrading capacity from grass carp intestinal microbiomes. *FEMS Microbiology Ecology* **91**: fiv107, **doi**: http://dx/doi.org/10.1093/femsec/fiv107

Xia, J., Lin, G., Fu, G., Wan, Z., Lee, M., Wang, L., Liu, X. and Yue, G. (2014). The intestinal microbiome of fish under starvation. *BMC Genomics* **15**:266, **doi**: 10.1186/1471-2164-15-266

Xing, M., Hou, Z., Yuan, J., Liu, Y., Qu, Y. and Liu, B. (2013) Taxonomic and functional metagenomics profiling of gastrointestinal tract microbiome of the farmed adult turbot (*Scopthalmus maximus*) *FEMS Microbiology Ecology* **86**, 432-443

Yan, Q.Y., van der Gast, C.J. and Yu, Y.H. (2012) Bacterial community assembly and turnover within the intestines of developing zebrafish. *PLOS ONE* **7**, e30603

Zarkasi, K., Abell, G., Taylor, R., Neuman, C., Hatje, E., Tamplin, M., Katouli, M. and Bowman, J.P. (2014) Pyrosequencing based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system. *Journal of Applied Microbiology* **117**, 18-27

Zhou, Z., Liu, Y., Huang, G., He, S., Shi, P., Yao, B. and Ringø, E. (2011) Do stocking densities affect the gut microbiota of gibel carp (*Carassius auratus gibelio*) cultured in ponds? *Journal of Aquaculture Research and Development* **S1**: 003, **doi**: 10.4172/2155-9546.S1-003

Appendix

A. Publication 1

Exploring the microbial diversity of the distal intestinal lumen and mucosa of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum) using next generation sequencing (NGS)

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Abstract

In this study, next generation sequencing (NGS) was used to survey the 16S rRNA ribotypes of the distal intestinal lumen and mucosal epithelium of farmed rainbow trout. This approach yielded a library consisting of 2 979 715 quality filtered paired sequences, assigned to genus level of taxonomy using the Ribosomal Database Project (RDP). A high level of diversity was observed in both regions. A total of 90 bacterial genera were identified in the lumen of all fish sampled, compared with 159 in the mucosa. The allochthonous microflora was dominated by sequences belonging to the γ Proteobacteria (mean sequence abundance 54.3%), in particular the Enterobacteriaceae, with *Yersinia*, *Serratia*, *Hafnia* and *Obesumbacterium* the most abundant genera. Fewer γ Proteobacteria (mean sequence abundance 37%) were present in the mucosa, and autochthonous communities consisted of a more even split among the bacterial classes, with increases in sequences assigned to members of the β Proteobacteria (mean sequence abundance 18.4%) and Bacilli (mean sequence abundance 16.8%). The principal bacterial genera recorded in the mucosa were *Cetobacterium*, *Yersinia*, *Ralstonia*, *Hafnia* and *Carnobacterium*. The results of this study demonstrate that the luminal and mucosal bacterial communities may be different in their respective structures, and that the mucosal microflora of rainbow trout may be more diverse than previous research has suggested. This research also demonstrated a degree of conservation of bacterial genera between individual fish sampled, and is to the author's knowledge the first time the MiSeq® NGS platform has been used to explore the rainbow trout intestinal microflora.

Keywords: aquaculture, bacteria, intestinal microflora, rainbow trout, 16S rRNA sequencing

Introduction

The skin, gills, eggs and intestinal tracts of fish all harbour bacterial communities that are thought to impact upon their overall health through their interaction with these tissues. The gastrointestinal (GI) tract in particular possesses a hugely diverse microbial ecology that appears to vary among different fish species (Austin & Austin 1987; Cahill 1990; Ringo, Strom & Tabachek 1995; Merrifield, Burnard, Bradley, Davies & Baker 2009; Wu, Gao, Zheng, Wang, Cheng & Wang 2010; Di Maiuta, Schwarzentruber, Schenker & Schoelkopf 2013). In carnivorous fish, the distal intestinal region has been studied more extensively as it is considered to be the primary site of nutrient absorption in the fish gut. This region is also believed to consist of a more stable microbial flora, owing to a lower level of exposure to bile salts, peristalsis, gastric acidity and digestive enzymes than the proximal region (Ringo, Olsen, Mayhew & Myklebust 2003; Hartviksen, Vecino, Ringo, Bakke, Wadsworth, Krogdahl, Ruohonen & Kettunen 2014). The extent to which this microflora differs among apparently healthy individuals has been accurately characterized in humans (Guarner & Malagelada 2003;

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B. Publication 2

Effects of low-level dietary microalgae supplementation on the distal intestinal microbiome of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum)

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Abstract

In this study, high throughput 16S rRNA sequencing was used to investigate the effect of a novel whole-cell dietary microalgae meal (*Schizochytrium limacinum*), on the distal intestinal microbiome of farmed rainbow trout, *Oncorhynchus mykiss*. Heterotrophic microalgae are rich in omega 3 polyunsaturated fatty acids, can be produced sustainably and have been shown to have beneficial effects on host health. After a 15-week trial period, microbial community profiles were compared between the distal intestinal contents of fish fed either a control diet or a treatment diet that partially replaced fish oil with microalgae meal, at a substitution level of 5%. The results of this research showed that the microbial communities of both fish populations were composed of similar microbial taxa, however, the treatment group fed the microalgae supplement possessed a greater level of microbial diversity than those in the control group. A limited number of bacterial taxa were discriminatory between diets and were significantly elevated in the treatment group, notably operational taxonomic units (OTUs) assigned to the genera *Streptococcus, Leuconostoc, Lactobacillus, Lactococcus* and *Weissella*. However, the overall structure of the intestinal microbiome between control and treatment groups was not found to be significantly different. The treatment group displayed a heavier mean weight and condition factor at the end of the trial period. The results of this study suggest that the tested microalgae meal can be used as a replacement for a proportion of fish oil in

aquafeeds, with minor changes to the intestinal microbiome of farmed rainbow trout, and positive effects on growth.

Keywords: aquaculture, bacteria, intestine, microalgae, microbiome, rainbow trout

Introduction

Numerous studies have reported that diet type is a major driver in shaping the bacterial communities of the gastro-intestinal (GI) tract, commonly referred to as the 'microbiome', of both terrestrial and aquatic animals (Buddington, Krogdahl & Bakke-McKellep 1997; Ringo & Olsen 1999; Merrifield, Dimitroglou, Foey, Davies, Baker, Bogwald, Castex & Ringo 2010; Dimitroglou, Merrifield, Carnevali, Picchietti, Avella, Daniels, Guroy & Davies 2011; Merrifield, Olsen, Myklebust & Ringo 2011; Gatesoupe, Huelvan, Le Bayon, Sevre, Aaasen, Degnes, Mazurais, Panserat, Zambonino-Infante & Kaushik 2014; Kormas, Meziti, Mente & Frentzos 2014; Miyake, Ngugi & Stingl 2015). These microbes are believed to play important roles in host development, immunity, digestion and nutrition (Romero, Ringø & Merrifield 2014). The impacts of novel dietary ingredients on the intestinal microbiome of farmed salmonids, in particular those of probiotics, prebiotics and immunostimulants, are receiving more attention as aquafeed formulations evolve in line with restrictions on antibiotic use, and the diminishing availability of natural sources of marine fishmeal and fish oil (Tacon & Metian 2008). The vast majority of these investigations

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